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Efficient interfacially-driven vehiculization of corticosteroids by pulmonary surfactant

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ABSTRACT

Pulmonary surfactant is a crucial system to stabilize the respiratory air-liquid interface. Furthermore, pulmonary surfactant has been proposed as an effective method for targeting drugs to the lungs. However, few studies have examined in detail the mechanisms of incorporation of drugs into surfactant, the impact of the presence of drugs on pulmonary surfactant performance at the interface under physiologically-meaningful conditions, or the ability of pulmonary surfactant to use the air-liquid interface to vehiculise drugs to long distances.

This study focuses on the ability of pulmonary surfactant to interfacially vehiculize corticosteroids such as beclomethasone dipropionate (BDP) or Budesonide (BUD) as model drugs. The main objectives have been to (a) characterise the incorporation of corticosteroids into natural and synthetic surfactants; (b) evaluate whether the presence of corticosteroids affect surfactant functionality; and (c) determine whether surfactant preparations enable the efficient spreading and distribution of BDP and BUD along the air-liquid interface. We have compared the performance of a purified surfactant from porcine lungs and two clinical surfactants: Poractant alfa, a natural surfactant of animal origin extensively used to treat premature babies, and CHF5633, a new synthetic surfactant preparation currently under clinical trials. Both, natural and clinical surfactants spontaneously incorporated corticosteroids up to at least 10% by mass with respect to phospholipid content. The presence of the drugs did not interfere with their ability to efficiently adsorb into air-liquid interfaces and form surface active films able to reach and sustain very low surface tensions (<2 mN/m) under compression-expansion cycling mimicking breathing dynamics. Furthermore, the combination of clinical surfactant with corticosteroids efficiently promoted the active diffusion of the drug to long distances along the air-liquid interface. This effect could not be mimicked by vehiculisation of corticosteroids in liposomes or in micellar emulsions similar to the formulations currently in use to deliver anti-inflammatory corticosteroids through inhalation.

KEYWORDS

Respiratory nanomedicine; Air-liquid interface; Lung surfactant; Drug delivery; Corticosteroids

INTRODUCTION

Pulmonary surfactant (PS) is a crucial system to stabilize the respiratory air-liquid interface. The alveoli of mammalian lungs, the place where gas exchange occurs, are very delicate structures that are constantly expanding and compressing to inhale fresh air enriched with oxygen and exhale the residual air containing carbon dioxide. PS reduces the surface tension at the thin aqueous layer coating the alveolar surface to values below 2mN/m, preventing pulmonary collapse during expiration and so reducing the work of breathing [1, 2]. The continuous compression/expansion cycling forces PS to adapt to such highly dynamic environment, promoting the PS interfacial films to fold and form three-dimensional phases while excluding certain lipid/protein complexes and any other non-compressible molecules from the interface, such as inhaled drugs or nanocarriers. PS also maintains a proper fluid balance in the lung, especially across the alveolar-capillary membrane, acts as a local barrier against infection and transports mucus and inhaled particles, impeding their adhesion to the upper airways [2, 3]. To fulfil these functions, surfactant has to adsorb very rapidly (in a few seconds) into the air-liquid interface and, once there, spread efficiently along it. As a consequence, PS offers unique opportunities to solubilize and vehiculize hydrophobic drugs, while protecting them from lung clearance.

The development of innovative drug delivery strategies is essential to increase the efficiency and efficacy of therapies, while targeting drugs to precise locations avoiding systemic side effects. More than 90% of new drugs are poorly soluble in water and are classified as class II or IV under the Biopharmaceutical Classification System (BCS). Consequently, the investigation of new strategies to facilitate solubilization of new generation drugs and the establishment of novel sites for drug entry are acquiring a high importance nowadays [2]. The respiratory system is becoming of particular relevance for the administration of different therapeutic agents not only for local action but also intended for systemic treatments. It constitutes a promising non-invasive alternative to other delivery routes, especially the intravenous one. The advantages of lungs compared to other drug delivery targets are their large surface area ($100m^2$ approx.), the high permeability of their membranes and the large vascularity, defined by the thin alveolar epithelium coating airways. Besides, due to the low enzymatic activity, clearance of drugs and nanoparticles is very slow [4]. Consequently, absorption and bioavailability of different types of molecules through this route are considerably higher than through conventional ways (oral, topical or intravenous), especially those that are poorly water-soluble.

The idea of using the PS system as a vehicle to transport therapeutic agents has been developed since the late 90's. From the first studies showing that PS could be a good delivery system for tobramycin [5, 6], many other molecules such as antibodies [7], corticosteroids [8], recombinant adenoviral vectors [9] or antioxidant enzymes [10] have been evaluated upon incorporation into surfactant-mimicking systems. In the present work, we have approached the incorporation and vehiculization of corticosteroids such as Beclomethasone dipropionate (BDP) or Budesonide (BUD) into pulmonary

surfactant, as a model of surfactant-promoted drug delivery. These compounds are anti-inflammatory steroids used to treat different respiratory diseases, such as asthma and bronchopulmonary dysplasia [11, 12, 13]. In particular, the combination of BDP and PS has shown synergistic effects to reduce certain markers of inflammation [14], improving respiratory function in preclinical models [15]. Moreover, clinical pilot studies utilized intra-tracheal instillation of BUD suspended in PS to improve the delivery of BUD to the lung periphery for the prevention of bronchopulmonary dysplasia (BPD). Yeh *et al.* reported a substantially reduced incidence of BPD with no observed immediate or long-term adverse effects [16]. For local action in the lungs, BDP or BUD are currently delivered in the form of aerosol, but new systems are needed to reduce the systemic exposure, the lung clearance and the total dosage, as well as to reach other target locations such as distal airways more efficiently. Nowadays several clinical surfactants obtained from bovine and porcine sources are being used to treat preterm new-borns [2, 17], and new synthetic preparations are under development [18, 19]. Therefore, it is crucial to investigate and compare the abilities of clinical surfactants of different nature to transport drugs as these corticosteroids.

This study addresses the ability of pulmonary surfactant to vehiculize corticosteroids trough the air-liquid interface, focusing on (a) characterising the incorporation of both corticosteroids into natural and synthetic surfactants; (b) evaluating whether the presence of corticosteroids affect surfactant functionality; and (c) determining whether surfactant preparations enable the distribution of BDP and BUD along the air-liquid interface. A surfactant preparation produced from minced porcine lung tissue, and CHF5633, a fully synthetic surfactant containing surrogates of hydrophobic proteins SP-B and SP-C, have been selected and compared with whole native surfactant purified from porcine lung, with respect to their capabilities to incorporate and vehiculize BDP or BUD along the air-liquid interface. The interaction of BDP with PS membranes was first studied to optimize incorporation of corticosteroids into surfactant complexes. Then, the effect of BDP and BUD on the PS behaviour was analysed using a Captive Bubble Surfactometer. Finally, the spreading properties and corticosteroid transporting potential of PS was evaluated with selected drug/PS combinations applied in a novel setup that couples two traditional Wilhelmy troughs connected by an interfacial bridge.

MATERIALS AND METHODS

Pulmonary surfactant (PS) systems. Poractant alfa (Curosurf®), a modified natural surfactant produced by reconstitution of a hydrophobic lipid/protein fraction obtained from minced porcine lungs, was provided by Chiesi Farmaceutici (Parma, Italy). It contains polar lipids and the hydrophobic surfactant proteins (SP-B and SP-C), with no cholesterol, and has a phospholipid concentration of 80 mg/mL. CHF5633, a fully synthetic surfactant containing equimolar proportions of dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and analogues of the hydrophobic surfactant proteins SP-B (CWLCRALIKRIQALIPKGGRLLPQLVCRLVLRCS) and SP-C (IPSSPVHLKRLKLLLLLLLLILLLILGALLLGL) [19], was also provided by Chiesi, at a concentration of 80 mg/mL. Native pulmonary surfactant (NS) was isolated from bronchoalveolar lavage (BAL) obtained from porcine lungs, following a protocol optimized at the laboratory [20, 21, 22]. Organic extracts (OE) from NS, Poractant alfa or CHF5633 were obtained as described by Bligh and Dyer [23]. Firstly, proteins were flocculated by incubating the samples for 30 min at 37°C in the presence of chloroform and methanol in a 1:2:1 (v/v/v) chloroform/methanol/water ratio. To allow formation of two phases, new volumes of chloroform and water were added to the mixture and then centrifuged at 3000g and 4°C during 5 min. In order to retrieve the maximum amount of the PS components, successive lavages with chloroform were performed. After each lavage, the hydrophobic components of NS were collected from the organic phase at the bottom.

To prepare multilamellar vesicle (MLV) suspensions, an appropriate volume of a chloroform/methanol solution of the DPPC/POPG (7:3 w/w) mixture (lipids obtained from Avanti Polar Lipids Inc.), or of the organic extracted materials, was dried under a nitrogen stream and then under vacuum for two hours to remove chloroform traces. The dried films were then hydrated with a buffered solution (5 mM Tris, 150 mM NaCl, pH 7.4) at 45°C, a temperature above the melting temperature of all the lipids in the mixtures (T_m = 41°C for DPPC). The hydration was accomplished in one hour with vigorous shaking every 10 min. Large unilamellar vesicles (LUVs) of DPPC/POPG (7:3 w/w) were prepared by extruding the MLV suspensions several times through polycarbonate membranes of 100 nm pore size (Whatman® Nuclepore Track-Etched Membranes, 19mm diameter, 0.1µm pore) in a mini-extruder (Avanti Polar Lipids Inc.), to obtain a relatively homogeneous suspension of unilamellar vesicles of ca. 100nm diameter. The extrusion was also carried out at 45°C.

The concentration of phospholipid in different samples was evaluated following a protocol established by Rouser *et al.* [24] based on its mineralization to phosphate and detection of the latter by a colorimetric reaction.

Corticosteroids. BDP, in the form of powder, or taking part of the aqueous suspension Clenil®, was provided by Chiesi Farmaceutici (Parma, Italy). BUD powder was also obtained from Chiesi, and Budesonide (Pulmicort®) was purchased from Astra Zeneca (Södertälje, Sweden). To determine the amount of the steroid in the surfactant

membranes, an aliquot of 10µL of BDP- or BUD-containing surfactant was diluted in 1mL of methanol and the absorption the solution measured at 235nm (ε_{235}= 0.0704 $M^{-1}cm^{-1}$).

In order to incorporate the drug into the surfactant preparations, we used three different protocols. In a first type of experiments, the proper amount of corticosteroid, diluted in methanol, was dried on the wall of tubes under a nitrogen flow and then incubated for 30 min at 37°C, with stirring every 10 min in the presence of the different surfactants or lipid suspensions. Alternatively, BDP or BUD was directly incorporated into organic extracts from NS or Poractant alfa and the mixture was then dried and resuspended in saline as explained above. In a third alternative, BDP or BUD were incorporated into clinical surfactants Poractant alfa or CHF5633 upon incubation with clinical formulations of the corticosteroids such as Clenil or Pulmicort. For some experiments, Clenil-like corticosteroid emulsions were previously prepared by combining the excipients of Clenil to solubilize the proper amount of BDP, the mixture BDP/BANB (see below), or BUD.

Drug impact on surfactant properties.To evaluate whether the drugs affect surfactant function under physiologically-relevant conditions, we have evaluated the performance of NS, Poractant alfa and CHF5633 samples, as well as that of suspensions prepared from an organic extract of NS or Poractant alfa, in a captive bubble surfactometer (CBS), in the absence or in the presence of BDP or BUD, as explained elsewhere [25].

The CBS is a device which allows evaluation of the PS activity at the surface of a millimetre-sized air bubble subjected to cyclic compression-expansion mimicking breathing dynamics, at physiological conditions of temperature, humidity and pH [26]. To measure the surfactant activity, a 200nL volume of each sample at a lipid concentration of 25mg/mL was injected into a buffered subphase containing sucrose to increase density (150 mM NaCl, 10% sucrose, 5 mM Tris, pH 7.4) inside a cylindrical sealed thermostatised chamber. The injection takes place close to the interface of a small air bubble (around 4-5 mm diameter), settled on the base of an agarose cap. The bubble can then be compressed and expanded by mean of a piston driven by a computer-controlled motor (see Fig. 1). Volume, surface area and surface tension can be calculated and monitored along the whole experiment by measuring the height and the diameter of the bubble recorded by a video camera [25, 27].

Interfacial drug vehiculization. In order to evaluate the ability of the different pulmonary surfactant preparations to efficiently vehiculize the corticosteroids along the air-liquid interface, a novel setup was designed in our laboratory based on Yu and Possmayer's design [28]. It consists of a double surface trough connected by an interfacial bridge (a wet No. 1 Whatman filter paper). Each surfactant sample, with or without drug, was deposited by spreading a small volume (15µL at 50mg/mL) of the suspension onto the interface of the donor trough (300mm^2). The adsorption into the interface and the diffusion through the interfacial bridge to reach the recipient trough

was monitored by following the changes in surface pressure (or surface tension) in both donor and recipient troughs (see Fig. 2). In these experiments, the subphase was not stirred, in order to maximize the differences in adsorption/spreading between the different materials. Subphase in the two troughs consisted in buffer 5 mM Tris, 150 mM NaCl, pH 7.4.

To follow the parallel diffusion of corticosteroid between donor and recipient troughs, a far-red fluorescent-labelled derivative of BDP (BANB, Fig. 3) was prepared. The synthesis of BANB involves saponification of BDP into beclomethasone, followed by oxidation of the latter with periodic acid to a carboxylic acid derivative (BC, Fig. 3). Finally, conjugation of BC to an amine derivative of the strongly fluorescent dye NileBlue (ANB) is carried out by amidation, after activating the BC with a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 1-hydroxybenzotriazole to provide the beclomethasone-Nile Blue conjugate (BANB, Fig. 3). BANB was purified by column chromatography on silicagel upon eluting the raw product with a chloroform/methanol/NH_4OH (6:1:0.1 v/v/v) mixture. Full experimental details and spectroscopic data of the above mentioned intermediates and BANB are provided in the Supplementary data section. The fluorescence was measured in samples taken from both donor and recipient troughs using an Aminco Bowman Series 2 spectrofluorometer.

RESULTS

Incorporation of corticosteroids into surfactant membranes

In order to evaluate the amount of drug that the different lipids systems were able to incorporate, an identical amount of BDP (100µg) was dried on tubes and exposed to increasing total amounts of different lipid and lipid-protein suspensions, including NS and its organic extract, and MLVs and LUVs made of DPPC/POPG (7:3, w/w). After incubation at 37°C, as explained in Materials and Methods, the mixture was separated into two fractions: (a) the membrane suspension, bearing the solubilised drug and (b) the remaining drug on the tube walls. The amount of BDP was then evaluated by absorption measurements as described in the Materials and Methods section. Figure 4 represents the percentage of BDP that was found associated to the membrane lipids as a function of the amount of lipid. The fraction of drug associated with lipid membranes rose following a linear trend, while the drug that remained on the tube decreased with a similar slope.

To confirm whether the different lipid suspensions follow a similar tendency as an evidence of the drug incorporation into the membranes, different amounts of BDP were exposed to identical lipid concentration (20mg/mL) of the different surfactant materials. Figure 5A shows how each sample incorporates the drug following an apparent hyperbolic incorporation curve. At lower drug/lipid ratios, LUVs of DPPC/POPG apparently captured more drug compared with the other samples. Suspensions of NS or of its OE followed very simillar trends, but capturing slightly less drug than pure lipid unilamellar suspensions. MLVs, on the other hand, were the samples capturing less drug at low drug/lipid ratios, likely because only the outer layers are effectively exposed to the drug. However, at higher drug/lipid ratios, MLVs tend to exhibit similar drug loading capacities than the other analysed preparations. In Figure 5B, the incorporation curves of BDP incorporated into the clinical surfactants Poractant alfa and CHF5633, can also be compared. Interestingly, they exhibit similar ability to solubilize BDP than native surfactant or its organic extract, in both qualitative and quantitative terms. Selected control experiments exposing the surfactants to amounts of BUD revealed a similar ability to incorporate the two tested corticosteroids (not shown).

Impact of corticosteroids on surfactant functional properties

Once established that the drug interacts and is incorporated into surfactant complexes, we analysed the impact of the drug on the biophysical properties of surfactant to form and sustain surface active films under physiological constrains, using the CBS. At 10% BDP/lipid ratio, most surfactant preparations were above its 80% saturation limit and, therefore, this amount of corticosteroid was selected to analyse the effects of the presence of the drug on the interfacial behaviour of the different surfactants. In Figure 6 the activity of NS and its reconstituted OE is analysed and compared in the absence and in the presence of 10% BDP.

Both, native surfactant and its organic extract showed very good initial and post-expansion interfacial adsorption that was not substantially affected by the presence of

drug. This means that the presence of the corticosteroid into the surfactant complexes does not alter their ability to rapidly associate to the air-liquid interface, to transfer lipid surface active species to form an orientated surface film that reduces surface tension to equilibrium values around 22-24 mN/m (seen in initial adsorption kinetics), and to spread and replenish the interface from associated structures once it is expanded (assessed in post-expansion adsorption). The main differences introduced by the presence of BDP are observed at the quasi-static compression-expansion isotherms. In these experiments, compression-expansion cycles are applied to the bubble in discrete slow steps, intercalated by lapse periods of time during which the film can relax. The analysis of these isotherms brings information about compressibility properties and stability of the films. These films can reorganize or experiment three-dimensional transitions usually detected by the presence of plateaus at the isotherms. These reorganizations or plateaus, typically observed in the first cycles, would be only partially detected under the kinetically limited conditions imposed by rapid compression-expansion cycles mimicking breathing dynamics. Q-static isotherms displayed in Figure 6 show how the presence of the corticosteroid induces the manifestation of plateaus into the compression legs, and, as a consequence, isotherms that require larger area reductions before the minimal tensions are reached. Frequently, several slow compression-expansion cycles are required before the isotherms of the films containing BDP are stabilized to produce the lowest tensions with minimal area reduction (see the details of the Q-static isotherms of NS in the absence or presence of BDP at the supplementary Figure 1). The higher hysteresis of Q-static isotherms of NS and OE in the presence of BDP is likely associated with a compression-driven progressive exclusion of the drug from the interface, because the rapid dynamic compression-expansion cycles carried out afterwards produce non-hysteretic isotherms indistinguishable in the absence and presence of the drug.

In Figure 7 the functional behaviour in the CBS of the clinical surfactant Poractant alfa is evaluated in the absence or presence of the corticosteroids BDP or BUD. The figure also compares the effect of the drugs incorporated by the different protocols described in Methods. As observed in the adsorption and compression-expansion isotherms of NS and OE, the presence of any of the corticosteroid, incorporated by any of the tested protocols, minimally affected the performance of surface films by Poractant alfa once subjected to compression-expansion dynamics. Initial adsorption of Poractant alfa was slightly affected by the presence of BDP, particularly when the drug was premixed with the surfactant material in organic solutions, presumably because that maximizes the incorporation of the drug into deep regions of surfactant lipid-protein complexes. Initial adsorption was also somehow slightly slowed when BDP was incorporated into Poractant alfa as part of Clenil. This is likely a transient effect of Clenil excipients, as observed upon exposure of Poractant alfa to equivalent emulsions BDP-free Clenil excipients (not shown). However, in all the experiments, and once the interface of the bubble was opened, post-expansion adsorption was equally rapid in the absence and in the presence of the drug. Supplementary Table 1 (see supplementary data) summarizes the main parameters defining the activity of

Poractant alfa in the absence or presence of the corticosteroids, incorporated by the different protocols.

As illustrated in Figure 8, the reconstituted surfactant CHF5633 can also incorporate 10% by weight of BDP or BUD with respect to phospholipid without relevant impairment of its functional properties. Initial adsorption of CHF5633 alone does not often produce very low surface tensions at first instance. However, upon interfacial expansion, this surfactant almost instantaneously reduced surface tension to equilibrium tensions of aprox. 25 mN/m, indicating the ultimate efficient transfer of surface active lipids into the interface. CHF5633 films could then reduce surface tension further to very low values of around 2 mN/m upon repetitive compression-expansion cycling. It is remarkable that the compression-expansion isotherms of CHF5633 always exhibit a kink at around 15 mN/m, a hallmark due to its particular composition and structure. The kink is fully reproducible and stable over the repetitive cycles, indicating that this is likely associated with a compression-promoted structural transition of the material and not to the irreversible exclusion of some components during compression. Interestingly, corticosteroid-loaded CHF5633 samples exhibited improved interfacial adsorption from the initial contact with the air-water interface, likely as a consequence of the slight perturbation introduced by the incorporated drugs on membrane fluidity. Neither BDP nor BUD altered substantially compression-expansion isotherms of CHF5633 films, as observed in the quasi-static or dynamic Π-A isotherms in Figure 8 or in the summary of surface activity parameters in the supplementary Table 1 (see supplementary data).

Interfacial vehiculization of corticosteroids

To assess whether incorporation into surfactant allows an efficient transfer and spreading of the drugs, we used a novel custom-made experimental design. As described in the Materials and Methods section, we connected two different troughs (donor and recipient), each monitored by a surface pressure sensor, by an interfacial bridge. PS samples, in the presence or in the absence of the drug, were introduced into the donor trough, and following the changes in surface pressure in both troughs we could monitor adsorption and movement of any surfactant material through the air-liquid interface.

Figure 9 summarizes the adsorption/spreading isotherms of different materials from the donor trough, where they were originally introduced, to reach the recipient chamber via diffusion through the air-liquid interface.

When an aliquot of any of the two clinical surfactants tested, Poractant alfa or CHF5633, was deposited at the donor trough, an immediate increase of surface pressure up to around 35-38 mN/m was recorded. After a lag time of aprox. 3-5 min, surface pressure started to increase also at the recipient trough, once the material adsorbed into the air-water interface of the donor chamber diffused along the interfacial connecting bridge. The increase in pressure in the recipient trough was accompanied by a slight decay in pressure at the donor chamber, indicating the transfer of material between the two compartments. However, surface pressure at the

donor trough never dropped below 30 mN/m, indicating that transfer of material towards the recipient chamber was associated with a continuous adsorption and replenishment of further material from the donor subphase. At a given time during the experiment (indicated by an arrow in the panels of Figure 9), the interfacial material at the recipient trough was cleared by aspiration, which produced an immediate decay in surface pressure. However, this decay was rapid and subsequently compensated by new adsorption and transference from the donor trough. This π-t kinetics demonstrated that there is a rapid and continuous connection of the two chambers via the air-liquid interface, and that this interfacial space can be used for a rapid interface-driven spreading and diffusion of surfactant material. The rapid equilibration of surface tension between the two interconnected compartments suggests that it is rather due to differences in interfacial molecular densities and the concomitant tension gradient-promoted Marangoni flows, and not to the diffusion of the material along the subphase as it is embedded into the porous structure of the paper bridge. The interfacial transfer of material between the two troughs could be followed in a comparable way when either Poractant alfa or CHF5633 were introduced in the donor trough. Interfacial transfer kinetics of these two materials were not affected when either of the two clinical surfactants were preloaded with 10% of BDP (with 1 % (w/w) consisting of the fluorescent analogue BANB), confirming that the inclusion of the corticosteroids did not alter the ability of the surfactants to adsorb and spread along the interface. Interestingly, when the material spread at the interface of the recipient trough was collected and taken to the spectrofluorimeter, the fluorescence spectrum of the probe could be easily detected (see right panels in Figure 9), indicating that the drug had diffused from the donor to the recipient chambers following the surfactant. The lower panels in Figure 9 illustrate how the introduction in the donor trough of a pure lipid sample (DPPC/POPG 7:3 w/w liposomes) produced a marginal increase in pressure, as a consequence of a poor adsorption of the lipids in the absence of any surfactant protein. The incorporation of BDP into those liposomes did not produce any improvement in interfacial adsorption and, as a consequence, very limited diffusion of material from the donor to the recipient trough. Therefore, we could not detect the fluorescence of BANB originally incorporated into the liposomes once material from the interfacial recipient compartment was extracted. Similarly, BANB was not transferred from the donor into the recipient chamber when the drug was introduced as a solution in Clenil excipients, the typical formulation in which BDP is administered to patients.

DISCUSSION

Several studies have reported the benefits of administering clinical steroids in combination with PS [14, 29, 30]. However, until now the ability of different surfactant formulations to incorporate them, the potential impact of the drugs on PS function and its vehiculization capabilities along the air-liquid interface have not been investigated at length. The data presented here support the concept that, provided a proper incorporation, PS is able to efficiently transport corticosteroids to long distances using the interface as a 'shuttle', without losing their ability to produce and sustain very low surface tensions along breathing-like compression-expansion dynamics.

Surfactant formulations seem very efficient in solubilizing poorly soluble drugs such as corticosteroids. In fact, all the surfactant preparations tested here have shown affinity to incorporate drugs such as BDP or BUD, regardless of the way the drugs were presented to the surfactant complexes. Both natural and clinical surfactants induced favourable partition of corticosteroids from dry films at the walls of glass tubes into their phospholipid-based membranes, and incorporating the drugs from their clinical formulations like Clenil or Pulmicort, typically used to deliver inhaled corticoids. We presume that corticosteroid molecules, somehow similar to cholesterol, a natural component of pulmonary surfactant in most species, likely integrate into phospholipid surfactant membranes simulating the way how cholesterol distributes between liquid-disordered and liquid-ordered regions [31, 32]. Pulmonary surfactant composition and structures are prepared to accept a certain proportion of steroids, at least up to ca. 15-20% (by weight with respect to phospholipids), without any impairment in surfactant function, as it has been demonstrated in the past [33, 34, 35]. Our drug incorporation experiments determined that both natural surfactant and clinical surfactants like Poractant alfa or CHF5633 can readily incorporate up to 10% by weight of corticosteroids, constituting a very efficient potential vehicle to convert the drugs into a deliverable formulation. Porcine natural surfactant already contains around 5-8% of cholesterol [17], while Poractant alfa and CHF5633 are produced in the absence of cholesterol. Taking into account that we are incorporating around 10% of additional steroid, none of the tested surfactant loadings goes beyond the potentially deleterious ratio of 20% steroid [33]. Previous studies, however, demonstrated that corticosteroids such as BUD are less deleterious for pulmonary surfactant function than cholesterol[36]. The group of Zuo also reported that a clinical natural surfactant such as Infasurf could incorporate up to 10% of BUD or BDP without significant functional effects[37].

Detailed functional analysis in the captive bubble surfactometer, under conditions simulating the demanding conditions imposed by respiratory mechanics, confirm that corticosteroid incorporation does not alter the main properties of porcine natural surfactant or those of the clinical preparations, Poractant alfa and CHF5633. The three pulmonary surfactant preparations tested exhibit good interfacial adsorption, both upon initial contact with the air-liquid interface or upon expansion of an interface previously exposed to the surfactants, once loaded with the drugs. The synthetic surfactant CHF5633 even shows better initial adsorptive properties in the

presence of BDP or BUD than in their absence. We think this is consistent with a fluidifying effect of the drug on the surfactant membranes, in a similar manner to what could be caused by a small percentage of cholesterol, thus promoting a more dynamic behaviour to interact and transfer surface active phospholipid molecules from the beginning, even before such dynamic transfer could be induced by an expanding interface. The main difference in the surface behaviour of corticosteroid-loaded surfactants compared with the corresponding drug-free preparations can be observed at the quasi-static compression expansion isotherms. In these experiments, a very slow compression can allow the reorganization of surfactant films to rearrange the distribution of the components that are less stable at the highest compressions, when the films are forced to adopt very high molecular packing. It seems that surfactant films, particularly those formed by the native surfactant, undergo a more extensive compression-driven reorganization in the presence of drugs, as noticed from the frequent larger plateaus, inducing an expansion of the Q-static isotherms to large compression rates. For instance, NS films require 40-45% area reduction during the first Q-static compression to reach minimal surface tension, whilst they reach a similar minimal tension with less than 20% area reduction in the second compression cycle. In contrast, and once loaded with 10% BDP, NS films require >50% area reduction to reach minimal tension in the first Q-static cycle, 40% in the second, and 25% in the third one. Only after four slow compression-expansion cycles the film is competent to produce minimal surface tension with less than 20% area reduction (see Figure 6). The increase in the hysteretic behaviour of NS films cycled at slow speed when the surfactant is bearing the corticosteroid could be interpreted as a consequence of the larger area reduction required during compression to produce the squeeze-out of the drug to surface-associated compartments that are likely out from the interface, and therefore do not interfere with reaching the maximal packing required to produce minimal tension at the end of compression. The drug could then be excluded from the interface in a similar manner to some of the natural surfactant components, such as unsaturated lipids or cholesterol [2, 38]. A similar behaviour has been also reported for NS films incorporating cholesterol or other spurious components such as those from meconium[38, 39]. Interestingly, both clinical surfactants Poractant alfa and CHF5633 do not show so marked increase in hysteresis as observed in the Q-static isotherms of NS films. We think that this difference could be due to the different intrinsic content of cholesterol. The larger exclusion plateaus in NS isotherms could be a consequence of a proportion of steroid near to the limit accepted by films compressed at tight packing. Clinical surfactants films, with no cholesterol, could still accept 10% of corticosteroid at their maximal pressures (minimal tensions).

The innocuous effect of corticosteroids such as BDP or BUD on the dynamic surface properties of PS complexes open new opportunities to use corticosteroid-loaded clinical surfactants as a basis of inhalable delivery strategies. Our double trough experiments demonstrate that corticosteroid-loaded surfactants can take the drug with them to adsorb onto peri-interfacial compartments, promoting its vehiculization to long distances through diffusion along the air-liquid interface. Surfactant could then

be prepared to use the interface as a 'shuttle' to spread the drugs. This might change, in our opinion, the potentially exploitable concept of inhalative strategies intended to deliver drugs through the pediatric or neonatal airways [2]. Traditional drug delivery through the airways has been usually linked to the production of small enough vehiculizable entities, such as aerosolized or nebulized particles of less than 2 μm of diameter [40]. According to that idea, only the smallest particles, propelled with enough energy, could be able to reach the narrowest distal airways and the alveoli. This could be particularly important for drugs intended for a lung-mediated systemic delivery, because the alveolar-capillary barrier is the surface exhibiting more favourable transport capabilities. However, we show here that if drugs are properly integrated into a clinical PS, in the right amounts and using procedures that preserve the most favourable surfactant dynamic properties, drugs could readily adsorb into the air-liquid interface and along it, travel from the upper to the distal airways to efficiently spread along the whole lung.

Several considerations have to be taken into account to extrapolate the concept of interfacially-driven drug vehiculization as derived from our experiments to a feasible and efficient pulmonary drug delivery. A first relevant question is that an eventual pulmonary delivery of clinical surfactant/drug combinations will not encounter an empty clean interface at the lung, but an interface that will be already occupied by different materials, such as endogenous surfactant, or that would be extended on top of a thick mucus layer at the upper airways. Additional experiments should be carried out to analyse how the spreading of surfactant-loaded drugs along the interface is modulated in a surfactant-saturated interface, or in the surface of complex solutions with a viscosity such as that induced by mucus [41]. On the other hand, the flow of fluid and particles into the upper airways is much influenced by the active participation of the mucociliary escalator, in charge of clearing accidentally inspired and potentially noxious entities [42]. However, still under that particular environment, the fate of molecules firmly associated to the interfacial film likely differs from those moving independently. It has been widely demonstrated that exogenous surfactant delivered as an endotracheal bolus can efficiently distribute along the airways and reach alveoli. The interfacial diffusion of surfactant/drug formulations could also be affected by the presence in the subphase -and in the interface- of different substances released as a consequence of lung injury, such as serum, or inflammatory mediators, which may affect lung surfactant properties that are linked to interfacial adsorption and spreading. In this regard, the proper combination of an efficient surfactant and drugs could simultaneously address the intended pharmacological action plus the restoration of the surfactant biophysical function. However, pulmonary surfactant-assisted drug vehiculization in a properly aerated lung could be defined by different clinical surfactant constraints than those affecting exogenous surfactant therapies in cases of lung injury requiring restoration of lung surfactant function. To that end, the amounts of surfactant to be delivered and optimal surfactant/drug proportions should only preserve the ability of the combined formulation to distribute along the interface rapid and efficiently.

Another important aspect is that the interfacially connected double trough presented here does not take into account the compression-expansion cycling at which pulmonary surfactant is subsequently subjected during breathing. Preliminary studies carried out in our laboratory seem to indicate that the interfacial dynamics could favour even further the transference of material from the donor to the recipient trough, a feature that has still to be investigated in detail. Breathing cycles could therefore also contribute to enhance the diffusion of PS and PS-associated entities towards the alveolar region. It has been well established that the particular composition of PS, especially the presence of the hydrophobic surfactant proteins, optimizes the interfacial behaviour of surfactant films during compression/expansion dynamics and facilitates the spreading of exogenous PS. During compression, part of the material already at the interface folds down and is excluded from it to be recycled; during expansion, that squeezed out PS likely generates surface tension gradients. Leveraging such gradients, mostly towards the alveoli, and attending to the Marangoni effects, we speculate that exogenous surfactant and molecules and particles that are properly associated could hook the endogenous surfactant and spread independently of fluid flows induced by the ciliated bronchial epithelia. Interfacial surfactant-associated phenomena could therefore allow reaching the alveolar region, with surfactant sharing the trip with additional elements, such as allergens, drugs or nanocarriers. However, further studies are required to evaluate the potential effect of fluid flows induced by the mucociliary escalator and the contribution of compression/expansion cycling dynamics occurring at the recipient trough.

A last important consideration is related to how pulmonary surfactant is usually delivered in exogenous therapies. Restoration of impaired surfactant function is frequently performed by delivery of enough amounts of a clinical surfactant through endotracheal tubes in patients that are intubated as a consequence of their respiratory problems. Those patients might have better expectations if treated with a proper combination of surfactant and drugs. However, to use surfactant as part of a general strategy to facilitate efficient through-lung drug delivery, better non-invasive administration strategies are desirable, which still preserve the dynamic properties that make surfactant an extraordinary pharmacological agent with enormous therapeutic potential.

CONCLUSIONS

Pulmonary surfactant, a lipid-protein complex with high surface activity, is a promising vehicle for transporting drugs along the whole respiratory surface. In this study, it has been demonstrated that, apart from incorporating corticosteroids without affecting its functional properties and transporting the drug through the air-liquid interface, PS could also efficiently release the drug by way of compression-expansion dynamics. Hence, clinical PS could be the basis of ideal materials to vehiculize hydrophobic drugs along the respiratory surface and help them to reach deeper regions in the lung, including the alveoli, possibly following surface tension gradients

generated along the air-liquid interfase during the process of breathing. Once there, the normal breathing process might also facilitate a progressive exclusion of the drugs into the alveolar region, which would facilitate their uptake by macrophages and pneumocytes, preceding their local action or their potential diffusion into the bloodstream. From a pharmaceutical point of view, this brings a huge potential as a promising alternative to improve current strategies for respiratory drug delivery. However, further experiments are needed to confirm that (1) PS can actually transport the drug along a native pulmonary surface in vivo, (2) the drug can be released from the surfactant in the alveolar regions, and (3) the drug can act locally but also diffuse to bloodstream.

CONFLICT OF INTEREST DECLARATION

Fabrizio Salomone is a Chiesi Farmaceutici employee. Chiesi Farmaceutici supplied its own products (Poractant Alfa and CHF5633). Apart of this, the authors declare no other conflict of interests.

ACKNOWLEDGEMENTS

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

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FIGURE CAPTIONS

Figure 1

Operational scheme of captive bubble surfactometry (CBS) experiments

CBS experiments start with the injection of a small volume (typically 200 nL) of concentrated surfactant (25 mg/mL phospholipid) near the surface of a millimetre-sized air bubble formed in a thermostated chamber containing a buffered dense saline solution. After 5 min to allow for surfactant adsorption and others 5 min after expansion of the bubble to facilitate surfactant spreading to form a surface active surface film, the chamber is closed and subjected to compression-expansion cycling mimicking breathing dynamics.

Figure 2

Setup designed to assess interfacial vehiculization of surfactant-drug combinations

A small (donor) Teflon trough (1,8 mL subphase) monitored by a surface pressure sensor is connected by an interfacial filter paper bridge with a second (recipient) trough (8 mL subphase, 25 cm^2), probed by another pressure sensor. A volume of surfactant or surfactant/drug combination is introduced into the donor trough, whose adsorption at the interface can be registered through an increase in surface pressure. Interfacial diffusion to reach and saturate the interface of the recipient trough is detected by a subsequent increase in surface pressure detected by another sensor.

Figure 3

Synthesis of the far-red fluorescent derivative of beclomethasone tagged with pseudo Nile Blue (BANB).

Reagents and conditions: (i) $KHCO_3$, MeOH, 72 h, r.t.; (ii) H_5IO_6, dioxane/H_2O, r.t., 48 h; (iii) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC · HCl), amino pseudo Nile Blue (ANB) derivative, 1-hydroxybenzotriazole (HOBT), Et_3N, CH_2Cl_2, r.t., 24 h. Full experimental details and spectroscopic data of the intermediates and product are provided in the Supplementary data section.

Figure 4

Incorporation of BDP into surfactant lipid and lipid/protein suspensions

The proportion of corticosteroid (100 μg) incorporated (solid lines) and non-incorporated (dashed lines) from dried films into suspensions of NS, its organic extract, MLV or LUV suspensions made of DPPC/POPG (7:3, w/w), has been plotted as a function of the concentration of phospholipid. Lines represent the linear plots that better fit the experimental data.

Figure 5

Incorporation curves of different pulmonary surfactant materials by BDP

The capacity of different pulmonary surfactant lipid or lipid/protein suspensions to incorporate the corticosteroid has been assessed by plotting the amount of incorporated drug vs. the total amount of drug (drug/lipid weight ratio) at which the suspensions have been exposed. A) Incorporation curves for

NS, its organic extract and MLV and LUV pure lipid suspensions (DPPC/POPG, 7:3 w/w). B) Incorporation curves for clinical surfactants Poractant alfa and CHF5633. Lines represent the best fit of experimental data to a hyperbolic incorporation curve.

Figure 6

Effect of BDP on the functional behaviour of NS and its organic extract, as assessed in the CBS

γ-t interfacial adsorption kinetics are compared in the absence or presence of BDP, for suspensions of NS (above) and of its organic extract (below), during either initial (left) or post-expansion (center left) adsorption. Data are average and error bars the standard deviation upon averaging the results of three different experiments. Representative γ-A compression-expansion isotherms, obtained during slow quasi-static (center right) or rapid breathing-like dynamic (right) compression-expansion cycling are also compared in the presence or absence of the drug. In the graphs the four quasi-static (1^{st} to 4^{th} from left to right) and 1^{st}, 10^{th}, and 20^{th} dynamic cycles are compared.

Figure 7

Effect of corticosteroids on the functional behaviour of Poractant alfa

Interfacial adsorption either upon initial injection at the surface of an air bubble at the CBS or upon bubble expansion has been compared for Poractant alfa alone (first row) or loaded with 10% (w/w with respect to phospholipid) BDP (2^{nd}, 3^{rd} and 5^{th} rows) or BUD (4^{th} and 6^{th} rows) through different protocols. Plotted in the left panels are mean data with standard deviation after averaging three different adsorption experiments. Right panels show illustrative isotherms for cycles 1-4 under quasi-static and cycles 1, 10 and 20 under rapid dynamic compression-expansion regimes applied to the different films.

Figure 8

Effect of corticosteroids on the functional behaviour of CHF5633

Interfacial adsorption either upon initial injection at the surface of an air bubble at the CBS or upon bubble expansion has been compared for CHF5633 alone (first row) or loaded with BDP (2^{nd} row) or BUD (3^{rd}, 4^{th} and 5^{th} rows) through different protocols. Plotted in the left panels are mean data with standard deviation after averaging three different adsorption experiments. Right panels show illustrative isotherms for cycles 1-4 under quasi-static and cycles 1, 10 and 20 under rapid dynamic compression-expansion regimes applied to the different films. The amount of drug in the different experiments was 10% (w/w with respect to phospholipids) except for BUD incorporated from Pulmicort, whose proportion was 1%, the maximal amount allowed by the dilution of CHF5633 with Pulmicort to reach the operational surfactant concentration.

Figure 9

Pulmonary surfactant-assisted interfacial vehiculization of corticosteroids

Compared are adsorption/spreading isotherms of Poractant alfa (first row) and CHF5633 (second row) in the absence (left panels) or presence (central panels) of BDP/BANB (9% total drug/phospholipid, 1% fluorescent probe), as measured by the increase in surface pressure detected in the donor (black line) or in the recipient (grey line) trough of a setup such as the one illustrated in the upper cartoon. At the time indicated by the arrow, interfacial material was harvested by suction at the surface of the recipient

trough and further changes with time of surface pressure were continuously followed as additional material was diffusing again from the donor trough. Data represent mean and standard deviation after averaging three different experiments. Compared are also adsorption/spreading isotherms of BDP/BANB Clenil (3rd row), or of a sample of DPPC/POPG (7:3, w/w) LUV liposomes (4th row) in the absence or presence of 10% BDP/BANB. On the right, the fluorescence emission spectra are compared of samples taken from the recipient trough at the end of the experiments.

FIGURES

Figure 1

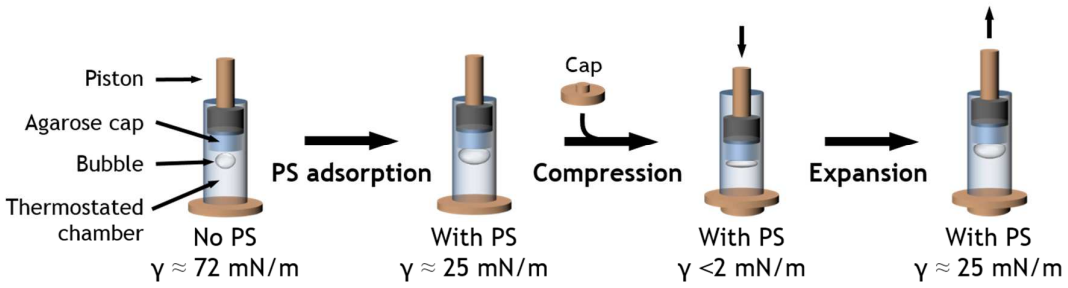


Figure 2

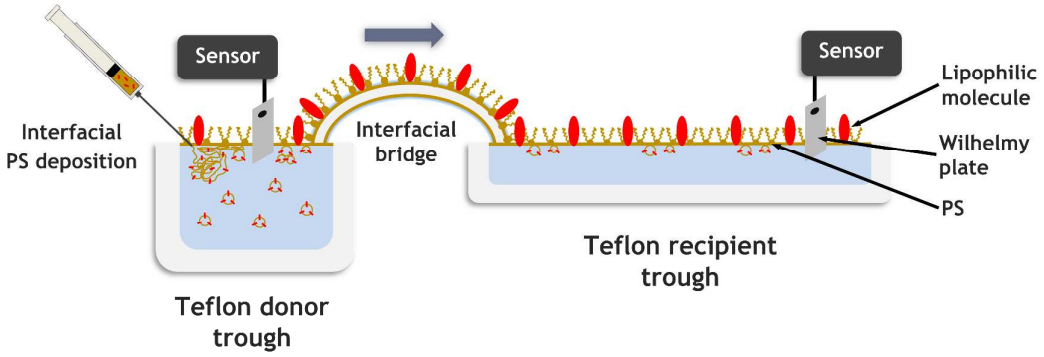

Sensor
Interfacial
PS deposition
Interfacial
bridge
Sensor
Lipophilic
molecule
Wilhelmy
plate
PS
Teflon donor
trough
Teflon recipient
trough

Figure 3

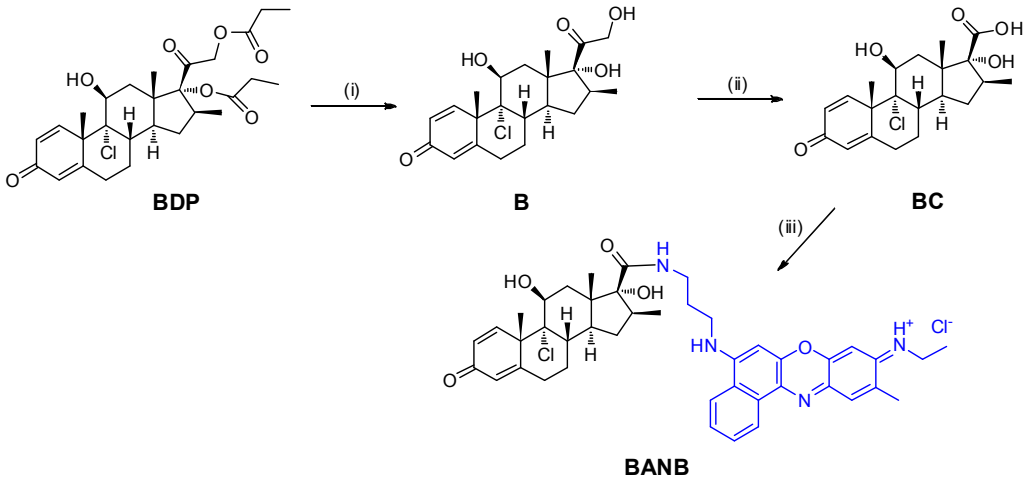


Figure 4

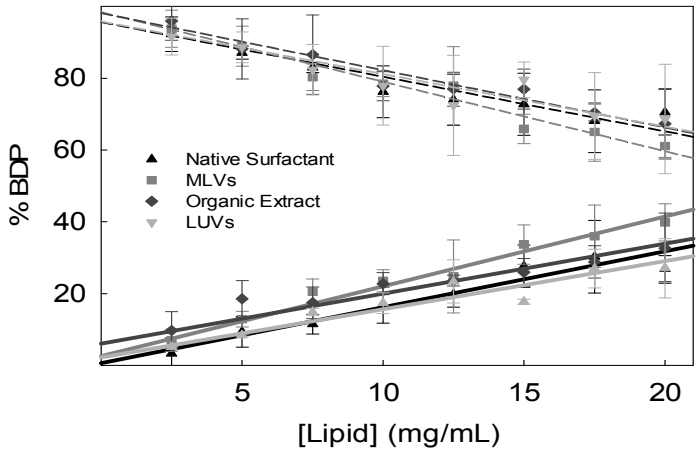

% BDP
80
60
40
20
5
10
15
20
[Lipid] (mg/mL)
Native Surfactant
MLVs
Organic Extract
LUVs

Figure 5

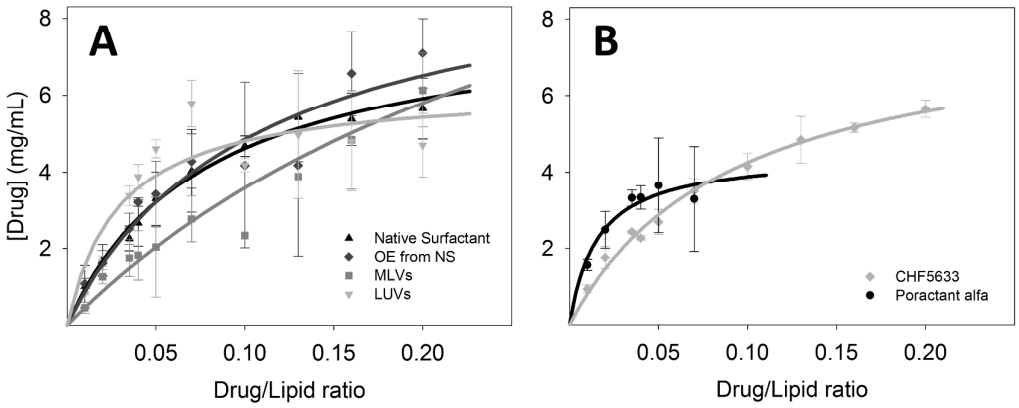

A
B
[Drug] (mg/mL)
Drug/Lipid ratio
Drug/Lipid ratio
Native Surfactant
OE from NS
MLVs
LUVs
CHF5633
Poractant alfa
2
4
6
8
0.05
0.10
0.15
0.20

Figure 6

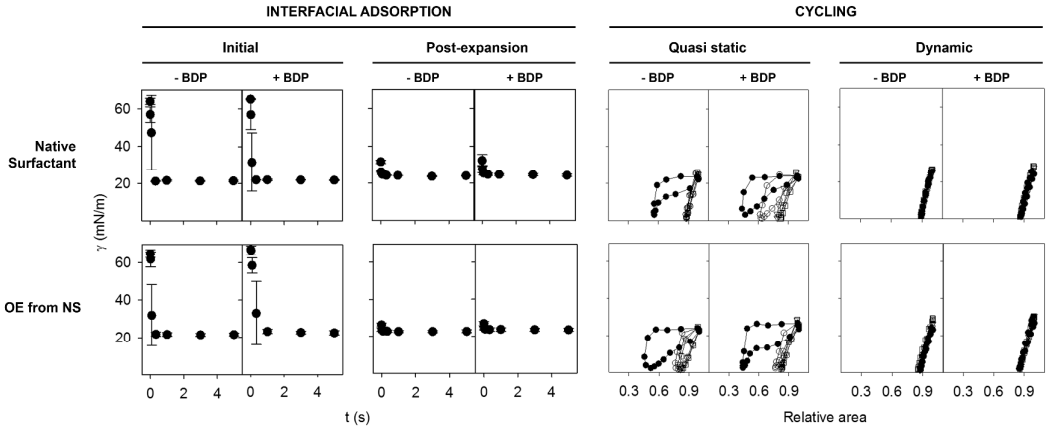

INTERFACIAL ADSORPTION
CYCLING
Initial
Post-expansion
Quasi static
Dynamic
- BDP
+ BDP
Native Surfactant
OE from NS
γ (mN/m)
t (s)
Relative area

Figure 7

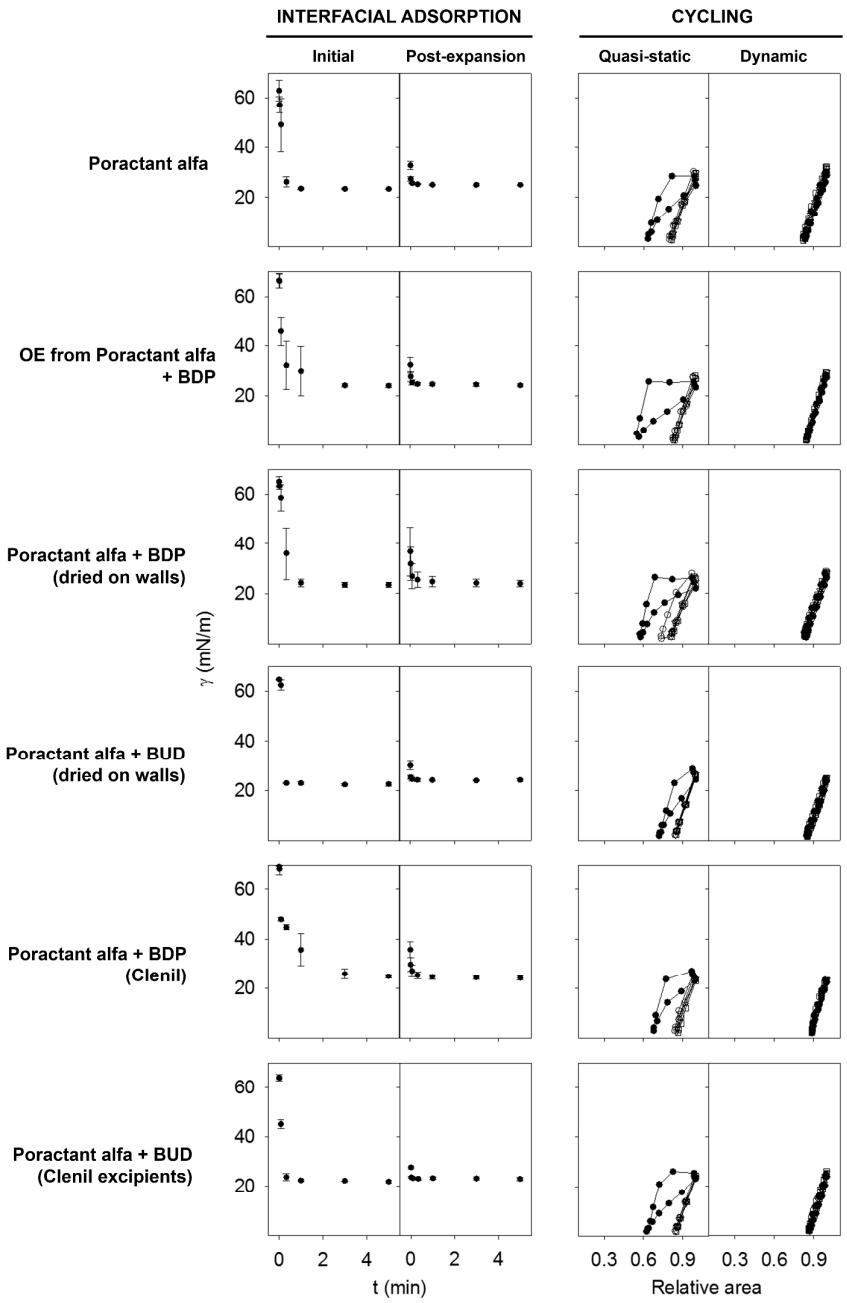

INTERFACIAL ADSORPTION
CYCLING
Initial
Post-expansion
Quasi-static
Dynamic
Poractant alfa
OE from Poractant alfa + BDP
Poractant alfa + BDP (dried on walls)
Poractant alfa + BUD (dried on walls)
Poractant alfa + BDP (Clenil)
Poractant alfa + BUD (Clenil excipients)
γ (mN/m)
60
40
20
0 2 4 0 2 4
t (min)
0.3 0.6 0.9 0.3 0.6 0.9
Relative area

Figure 8

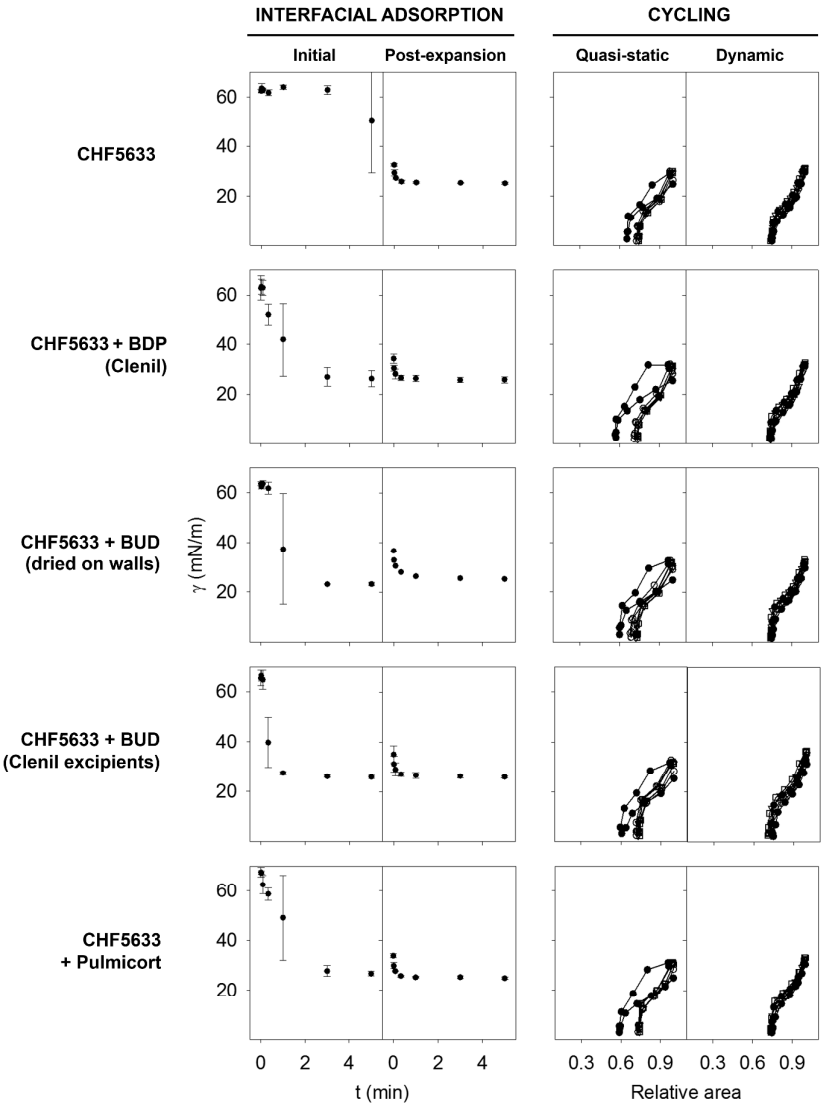
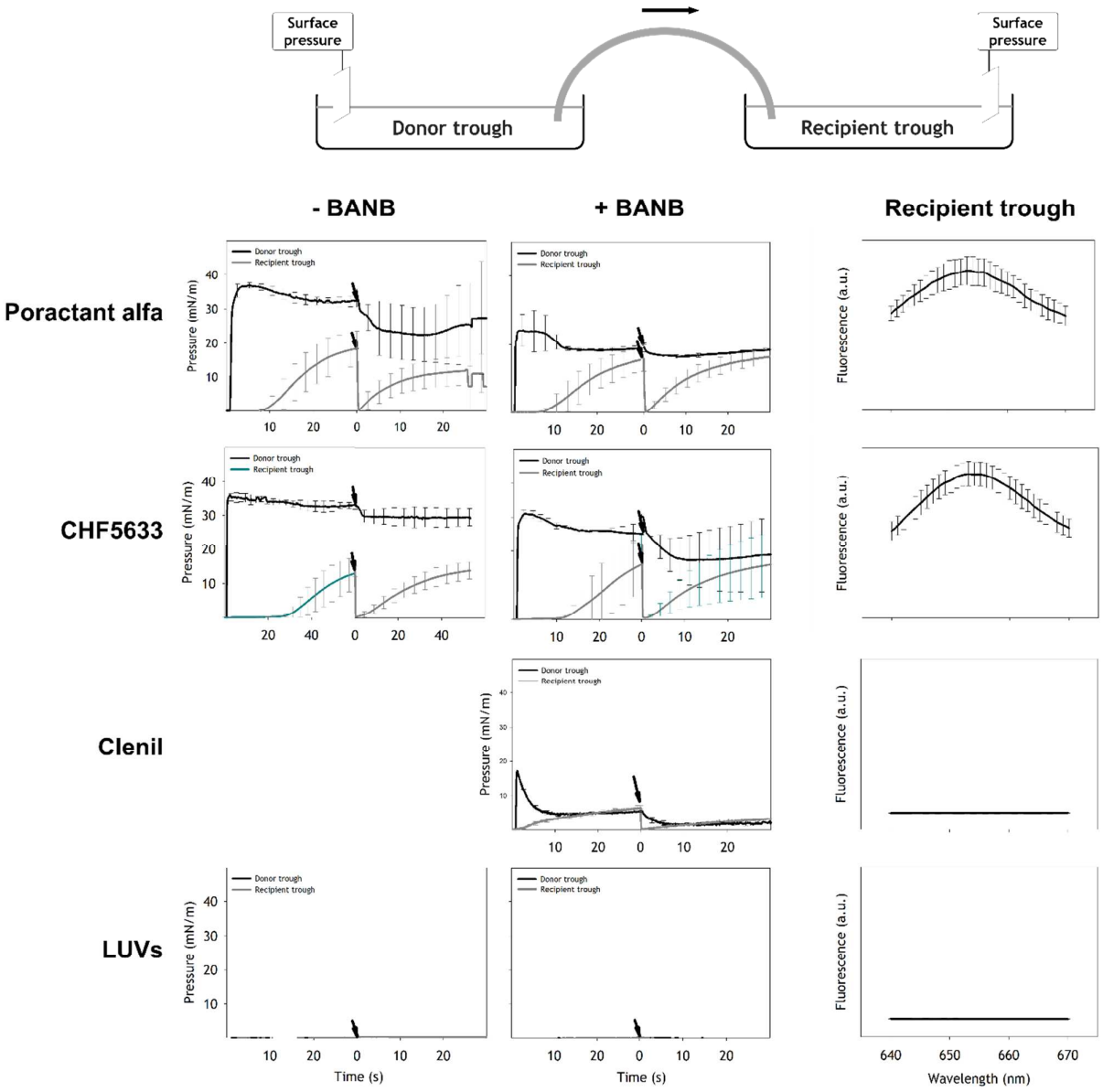

INTERFACIAL ADSORPTION
CYCLING
Initial
Post-expansion
Quasi-static
Dynamic
CHF5633
CHF5633 + BDP (Clenil)
CHF5633 + BUD (dried on walls)
CHF5633 + BUD (Clenil excipients)
CHF5633 + Pulmicort
γ (mN/m)
t (min)
Relative area

Figure 9


Surface pressure
Surface pressure
Donor trough
Recipient trough
- BANB
+ BANB
Recipient trough
Poractant alfa
CHF5633
Clenil
LUVs
Donor trough
Recipient trough
Pressure (mN/m)
Fluorescence (a.u.)
Time (s)
Wavelength (nm)

Graphical Abstract