Transient Exposure of Pulmonary Surfactant to Hyaluronan Promotes Structural and Compositional Transformations into a Highly Active State*

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Pulmonary surfactant is a lipid-protein complex that lowers surface tension at the respiratory air-liquid interface, stabilizing the lungs against physical forces tending to collapse alveoli. Dysfunction of surfactant is associated with respiratory pathologies such as acute respiratory distress syndrome or meconium aspiration syndrome where naturally occurring surfactant-inhibitory agents such as serum, meconium, or cholesterol reach the lung. We analyzed the effect of hyaluronan (HA) on the structure and surface behavior of pulmonary surfactant to understand the mechanism for HA-promoted surfactant protection in the presence of inhibitory agents. In particular, we found that HA affects structural properties such as the aggregation state of surfactant membranes and the size, distribution, and order/packing of phase-segregated lipid domains. These effects do not require a direct interaction between surfactant complexes and HA and are accompanied by a compositional reorganization of large surfactant complexes that become enriched with saturated phospholipid species. HA-exposed surfactant reaches very high efficiency in terms of rapid and spontaneous adsorption of surfactant phospholipids at the air-liquid interface and shows significantly improved resistance to inactivation by serum or cholesterol. We propose that physical effects pertaining to the formation of a meshwork of interpenetrating HA polymer chains are responsible for the changes in surfactant structure and composition that enhance surfactant function and, thus, resistance to inactivation. The higher resistance of HA-exposed surfactant to inactivation persists even after removal of the polymer, suggesting that transient exposure of surfactant to polymers like HA could be a promising strategy for the production of more efficient therapeutic surfactant preparations.

Significance:

Understanding the effects of polymers as additives in surfactant will allow the development of new therapeutic options.

Results:

Exposure to a hyaluronan meshwork modifies the structure and composition of surfactant.

Conclusion:

Transient exposure of surfactant to hyaluronan leads to a higher resistance to inactivation.

Background:

Pulmonary surfactant is inactivated under pathological conditions, making clinical surfactants fail in respiratory therapies.

Pulmonary surfactant is a complex mixture of lipids and proteins that lines the alveolar air-water interface. By lowering surface tension at the respiratory interface, pulmonary surfactant stabilizes air spaces by reducing the physical forces that tend to collapse alveoli. The surfactant system is composed of 90% lipids (mainly phospholipids) and four specific proteins (SP-A, SP-B, SP-C, and SP-D) that are synthesized, assembled, and stored in special compartments (lamellar bodies) within alveolar epithelial type II cells. The major component of surfactant is the disaturated phospholipid dipalmitoylphosphatidylcholine, which is also mainly responsible for lowering surface tension to near zero values during compression of the alveolar surface upon exhalation. Inactivation of surfactant is related to an impairment of its surface activity, resulting in abnormally high surface tension, which results in lung dysfunction. Inactivation of surfactant in the lungs is associated with respiratory pathologies such as acute respiratory distress syndrome and meconium aspiration syndrome where naturally occurring inhibitory agents such as serum, meconium, or cholesterol reach the lungs. In the case of meconium aspiration syndrome, surfactant used for treatment is inactivated, as is the endogenous surfactant (1). In acute respiratory distress syndrome, partial inactivation of exogenous surfactant also occurs (2). Therefore, enhancing surfactant resistance to inactivation may open the possibility of designing more efficient treatments for these pathologies.

Previous work by us and others (3–12) has shown that a range of polymers can enhance surfactant activity, among them hyaluronan (HA). HA is a naturally occurring linear polysaccha-

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ride with repeating disaccharide units of glucuronic acid and N-acetylgalactosamine, linked via alternating β 1–4 and β 1–3 glycosidic bonds. It is omnipresent in the extracellular space of vertebrates, e.g. in tissues such as cartilage, in synovial fluid, and also in the lungs. Type II pneumocytes secrete HA into the aqueous layer lining the respiratory epithelium (13, 14). In normal alveolar fluid, the average estimated molecular mass of HA is 220 kDa (15).

At physiological salt and pH, isolated HA polymer chains adopt the form of a swollen random coil in aqueous solution that is well hydrated and contains ~1000-fold more water than polymer (13, 16). As a consequence, the so-called overlap concentration, i.e. the solution concentration at which the individual polymer coils start to interpenetrate, is rather low: around 1 mg/ml, somewhat dependent on the polymer size (17). At concentrations above the overlap concentration, HA forms a continuous meshwork of dynamically interpenetrating chains that pervades the solution (18).

The rather unique space-filling properties of HA arise from the combination of a relatively stiff backbone and interchain repulsion (19). In addition to the large contribution that negatively charged carboxyl groups of HA make to interchain repulsion (19) and, hence, expansion of the coil and, thus, the persistence length, a measure for the stiffness of a polymer (13, 16). As a consequence, the so-called overlap concentration, i.e. the solution concentration at which the individual polymer coils start to interpenetrate, is rather low: around 1 mg/ml, somewhat dependent on the polymer size (17). At concentrations above the overlap concentration, HA forms a continuous meshwork of dynamically interpenetrating chains that pervades the solution (18).

Specific features observed in previous studies, such as the aggregation of surfactant complexes in the presence of polymers (4), led us to hypothesize that physical (i.e. entropy-driven) phenomena related to the formation of a meshwork through the interpenetration of polymer chains above the overlap concentration may exert effects on surfactant complexes independently of the chemical nature of the inactivating agent (7). Among these phenomena are osmotic stress and the so-called depletion force. The latter is an attractive force that occurs between colloids (in our case surfactant vesicles or complexes) in the presence of particles or a polymer meshwork if the size of the particles or the meshes, respectively, is smaller than the colloid size (6, 8, 9, 26, 27) and provided that the particles/polymers do not bind to the colloid. These physical forces may produce changes in the structure and composition of surfactant that may increase the resistance of surfactant to inactivation not only by serum proteins but also by membrane-disturbing substances such as cholesterol or meconium (7). Although such effects would be general for polymers such as HA, PEG, and dextran, the polymer concentration needed would be particularly low for HA because of its low overlap concentration. With its exceptionally low overlap concentration and biocompatibility, HA is an attractive candidate polymer with potential therapeutic applications to study such an effect.

In this work, we studied the structure and biophysical behavior of surfactant after transient exposure to HA in the presence or absence of inactivating agents. We provide evidence that physical effects pertaining to a polymer solution can generate biological function in a rather surprising way.

**EXPERIMENTAL PROCEDURES**

Native porcine lung surfactant (NS) was purified from bronchoalveolar lavage by NaBr density gradient centrifugation as described previously (8). NS was used in aqueous suspensions without organic extraction. NS contained the full complement of surfactant proteins SP-A, SP-B, and SP-C. Phospholipid concentration was measured by analysis of lipid phosphorous (28). The surfactant amounts and concentrations described are estimated from phospholipid mass, taking 750 Da as the average phospholipid molecular mass. Throughout this study, three different batches of NS were used, and all produced consistent results. Dilutions of material were made with 5 mM Tris (pH 7) buffer containing 150 mM NaCl. First-passed meconium from term infants was collected, pooled, and lyophilized. Water-soluble cholesterol (complexed with methyl-β-cyclodextrine) was obtained from Sigma (catalog no. C4951) and mixed (at a final proportion of 4% cholesterol w/w) with NS. We had determined previously that exposure of NS to cholesterol-loaded methyl-β-cyclodextrine increases the proportion of cholesterol from 3.1 ± 0.2% with respect to phospholipid mass to 5.8 ± 0.8% (29). Porcine serum was obtained from porcine blood and used at a protein concentration of 100 mg/ml. HA from *Streptococcus zooepidermicus*, 120 kDa on average, was obtained from Sigma (catalog no. H9390) and dissolved at different concentrations in buffer. To obtain surfactant that was transiently exposed to HA, we incubated surfactant and HA for 30 min. Thereafter, the mixture was centrifuged at 22,000 × g for 30 min, and surfactant was recovered within the pellet, whereas HA was retained in the supernatant.

**Hyaluronan-Surfactant Binding Assay by QCM-D**—The specific interaction of surfactant with HA was assayed by quartz crystal microbalance with dissipation monitoring (QCM-D) (30). Films of end-grafted hyaluronan were prepared on the surface of a silica-coated QCM-D sensor (QSX303, Biolin Scientific, Västra Frölunda, Sweden) adapting a method described previously (31). Briefly, HA with a molecular mass of 280 kDa and a biotin group at the reducing end (Select-HA B250, Hyalose, Oklahoma City, OK) was end-grafted to a neutravidin-coated, silica-supported lipid bilayer. The silica-supported lipid bilayer was formed from small unilamellar vesicles made from dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine-cap-biotin (DOPE-cap-biotin) (Avanti Polar Lipids, Alabaster, AL) (molar ratio 9:1) by the method of vesicle spreading (32).

Neutravidin served as a molecular linker, interconnecting biotin groups in the silica-supported lipid bilayer and on HA. The preparation of the HA film and its interaction with surfactant were monitored by QCM-D. Native surfactant complexes were applied at a concentration of 0.1 mg/ml in the form of multilamellar vesicles (MLVs).
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Langmuir–Wilhelmy Balance—In a Nima surface balance (200-cm² trough) equipped with a continuous Teflon ribbon barrier (Nima Technology, Coventry, UK), aqueous NS was spread onto the buffered subphase (5 mM Tris (pH 7) and 150 mM NaCl). After 10 min of equilibration time, the films were compressed at 60 cm²/min from a maximum area of 200 cm² to a minimum area of 20 cm². Surface pressure versus area isotherms are presented, with the surface area given in Å² per phospholipid molecule.

Formation and Imaging of Surfactant Giant Unilamellar Vesicles (GUVs)—NS labeled with rhodamine-DOPC and TOP-Fluor-cholesterol (0.1% mol of probe/mol of phospholipid each, added in a trace of dimethyl sulfoxide) at a phospholipid concentration of 2 mg/ml was applied in the form of drops on indium-tin oxide-coated glass slides and dried at 37 °C. Two indium-tin oxide-coated glass slides were then assembled into a capacitor-type configuration. The chamber was filled with buffer (5 mM Tris and 150 mM NaCl) and sealed with Teflon spacers and vacuum grease. The indium-tin oxide-coated glass slides were then connected to a pulse generator. We first applied an alternating voltage of 250 mV at a frequency of 500 Hz. The voltage was then increased by 250 mV every 5 min to 1.5 V. Subsequently, frequency and voltage were maintained at 500 Hz and 1.5 V, respectively, for 1 h. Finally, we decreased the frequency by 100 Hz every 10 min (33). GUVs were studied with an Axioplan II microscope (Zeiss, Dublin, CA) for epifluorescence imaging.

Fluorescence Spectroscopy—Emission spectra of rhodamine-DOPC or rhodamine-dipalmitoylphosphatidylethanolamine (DPPE)-labeled surfactant, used as model probes of unsaturated and saturated phospholipids, respectively, were taken in a spectrofluorometer (SLM-Aminco AB2, Urbana, IL). Emission spectra were recorded in the 570- to 640-nm range upon excitation at 560 nm.

Aggregation of Surfactant Large Unilamellar Vesicles—Large unilamellar vesicles (LUVs) were formed using a mini extruder (Avanti Polar Lipids) equipped with a polycarbonate membrane of 0.1-μm pore size. Suspensions of MLVs, formed by rehydration of dried lipid films or aqueous NS, were pressed 11 times through the extruder membranes, leading to the formation of unilamellar vesicles with a size comparable with that of the membrane pores. Aggregation of LUVs, alone or mixed rapidly with HA, was assessed by measuring the apparent increase in the absorbance at 400 nm because of turbidity during 20 min after LUV preparation.

Captive Bubble Surfactometry—Surfactant performance was evaluated in the captive bubble surfactometer operated at 37 °C as described previously (34, 35). The bubble chamber contained 5 mM Tris-HCl (pH 7), 150 mM NaCl, and 10% sucrose. After a small air bubble (0.035–0.040 cm³) was formed, ~150 nl of surfactant (10 mg/ml) was deposited below the bubble surface with the aid of a transparent capillary. Following the introduction of surfactant, the change in surface tension (γ) was monitored over 5 min from changes in the shape of the bubble (36). The chamber was then sealed, and the bubble was rapidly (i.e. within 1 s) expanded to 0.15 cm³ to record postexpansion adsorption. Five minutes after expansion, dynamic cycles were started in which the bubble was continuously compressed and expanded at 20 cycles/min. Data from initial and postexpansion adsorption are presented as averages ± S.D. of three experiments. Graphs plotting the dynamic cycles correspond to single representative experiments.

To study inactivation by serum, we injected serum and surfactant sequentially into the captive bubble surfactometer chamber, allowing surfactant to compete with a preformed interface of serum, as described previously (7). When testing inactivation by meconium or cholesterol, either meconium or cyclodextrin-solubilized cholesterol were first added to surfactant, and the mixture was reincubated for 30 min before being injected into the captive bubble surfactometer chamber (29). To study inactivation, we used a concentration of surfactant of 10 mg/ml, which exhibits susceptibility to inactivation, as described previously (7). In some experiments, HA was also premixed with surfactant and injected immediately, as described above.

RESULTS

The presence or absence of specific interactions between surfactant and HA determines the physical effects that HA can potentially exert on surfactant complexes. In particular, the lack of specific attractive interactions is a requirement for depletion forces to drive surfactant complex aggregation (37).

To test whether HA interacts specifically with surfactant complexes, we monitored surfactant binding to a HA-covered surface by QCM-D. This technique provides information about binding events and about the morphology and viscoelastic properties of surface-bound material through measuring changes in the resonance frequency (Δf) and dissipation (ΔD) of the QCM-D sensor. HA with a molecular mass of 280 kDa was bound through a biotin group at the reducing end to a sensor surface that was functionalized previously with a biotin-capturing biomolecular coating, i.e. a neutravidin-coated, silica-supported lipid bilayer (Fig. 1A). Because of the intrinsic stiffness of HA chains and interchain repulsion, a highly hydrated, surface-confined film (a so-called brush) with an HA concentration of a few milligrams per milliliter is formed (19, 31). We did not observe any significant QCM-D response when exposing surfactants in the form of MLVs to such a film (Fig. 1B) and conclude that there is no attractive interaction between surfactant complexes and HA.

It has been shown previously that interfacial adsorption of surfactant is enhanced in the presence of HA (8). Fig. 2 shows that the application of an aqueous suspension of NS together with HA at the aqueous surface in a Langmuir trough results in enhanced adsorption and transfer of material to the air-water interface as compared with NS alone. In fact, the transfer in the presence of HA was as efficient as the deposition of surfactant in the form of an organic extract directly at the air-water interface. It should be noted that the addition of HA to NS does not significantly modify the isotherm compared with that from the organic extract, indicating that HA is not occupying space at the interface. On the basis of the lack of direct interaction between NS and HA that we found in the QCM-D assay, we propose that HA diffuses away from the interface and becomes highly diluted in the large subphase of the trough right after application (to a final concentration of 0.0625 μg/ml). One
could assume that, instead of a specific biochemical interaction between the two materials, depletion forces may be acting to facilitate interfacial adsorption, as already proposed earlier by others (9). It is worth noting, however, that such forces could only act while surfactant and HA are mixed together and at the first instances after surfactant application to the air-water interface. When the HA has diffused into the subphase, the polymer concentration, and, hence, the depletion forces, would be very small.

The analysis of the morphology of surfactant membranes converted into GUVs as a model of surfactant bilayers allows the study of certain membrane properties, such as the lateral organization of lipid phases, and how this could be modified by the presence of HA in the bulk phase. As shown in Fig. 3, GUVs...
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GUV domain distribution  GUV size distribution

FIGURE 3. Effects of HA on phase segregation and size of pulmonary surfactant GUVs. Surfactant was labeled with rhodamine-DOPE (red) and TopFluor-cholesterol (green). Left, representative epifluorescence micrographs of a GUV from native surfactant in the absence (top panel) or presence (bottom panel) of 2.5 mg/ml HA in the bulk phase. Scale bar = 10 μm. Right, size distribution of vesicles obtained by quantification of the diameter of GUVs from surfactant in the absence (top panel) or presence (bottom panel) of HA in the bulk phase. n = 70 vesicles/group.

of NS had an average size of about a 12-μm diameter and displayed a rather dense and homogeneous distribution of relatively small, circular domains enriched in rhodamine-DOPE embedded in a continuous matrix enriched in TopFluor-cholesterol. Presumably, the circular domains are in the fluid liquid-disordered phase, whereas the matrix is a dipalmitoylphosphatidylcholine- and cholesterol-enriched liquid-ordered phase, as reported previously (38, 39). When HA was added to the bulk solution at a final concentration of 2.5 mg/ml, the appearance of the domains enriched in rhodamine-DOPE changed drastically. HA promoted the formation of fewer but larger domains. The domain sizes became more heterogeneous, and some domains appeared aggregated (Fig. 3A, bottom panel, arrow). There was also a clear decrease of the average size of the GUVs down to around 8 μm in diameter. It seems that the HA-promoted decrease in the size of surfactant GUVs induced redistribution of segregated domains and led to formation of membrane regions that protrude out of the GUVs (Fig. 3, left, bottom panel). These regions seem to be particularly enriched in the fluid-disordered membrane phase, suggesting that the detachment of some of these membrane patches could lead to a compositional refinement.

To test whether HA promotes refinement of the composition of native surfactant complexes, we included rhodamine probes, attached either to a saturated (rhodamine-DPPE) or to an unsaturated (rhodamine-DOPE) phospholipid species in MLV suspensions of NS. To separate large surfactant complexes from smaller, segregated domains or vesicles, we centrifuged the sample and resuspended only the pellet, where most of the large aggregates are deposited, thus eliminating small vesicles and most of the HA, which remained in the supernatant. Fig. 4 demonstrates that transient exposure of rhodamine-DPPE-labeled surfactant to different concentrations of HA does not cause significant changes in the fluorescence intensity of the sample, indicating that most of the labeled saturated phospholipids are retained in the pelleted large surfactant complexes. In contrast, transient exposure of rhodamine-DOPE-labeled surfactant to HA caused a substantial loss of fluorescence of the samples. Loss of the fluorescence probe is associated with surfactant pellets that were transiently exposed to HA concentrations of 1 mg/ml or more (Fig. 4, inflection point of the sigmoidal fit at 0.93 ± 0.04 mg/ml). This indicates that small protrusions of unsaturated lipids were segregated from the large aggregates and MLVs of surfactant and lost together with the HA in the supernatant. This HA-driven compositional refinement could be non-reversible, which could promote permanent changes in functional properties of surfactant. The dose-dependent effect of HA suggests that there is a critical concentration for HA to produce compositional refinement of unsaturated phospholipids. Interestingly, the concentration threshold of around 1 mg/ml corresponds to the overlap concentration above which HA chains in solution start to interpenetrate and form a continuous mesostructure.

We also determined the amount of cholesterol in surfactant pellets after exposure to HA and found a decrease in cholesterol associated with surfactant complexes exposed to high concentrations of HA in the bulk phase (Fig. 5, inflection point at 1.08 ± 0.18 mg/ml). This provides additional evidence for HA-driven compositional refinement.

It has been shown previously that exposure to HA induces aggregation of surfactant preparations (4). As deduced from the sigmoidal fit in Fig. 6, the threshold HA concentration required for aggregation of surfactant LUVs is 0.78 ± 0.54 mg/ml, i.e. again in the range of the HA overlap concentration. This suggests that the meshwork of HA may be directly responsible for the aggregation of surfactant complexes. This agrees with the observation of a macroscopic segregation of a surfactant-free space (4) where the HA meshwork could be placed. The HA meshwork would then exclude surfactant from its space, inducing a separation into two distinct phases, rich in surfactant and HA, respectively, and possibly compaction of surfactant.

We hypothesized that the structural and compositional changes induced by HA are non-reversible, i.e. that an “activated” state of surfactant is retained after removal of HA. To test this, we studied the interfacial adsorption of surfactant that was first exposed to and then depleted from HA with a Langmuir-Wilhelmy balance (Fig. 7). Indeed, transient exposure to HA at concentrations higher than 0.98 ± 0.04 mg/ml, as estimated from the fit of the experimental data to a sigmoidal curve, resulted in a significantly enhanced surfactant adsorption. This indicates that surfactant can be permanently activated by transient exposure to HA at concentrations above 1 mg/ml.

We tested surfactant transiently exposed to HA against serum and cholesterol inactivation using the modified protocol in the captive bubble surfactometer described previously (7). Fig. 8 shows how serum inactivates unprimed surfactant, as described previously, whereas surfactant that was activated by transient exposure to HA was able to adsorb to an interface saturated with serum proteins. Surfactant transiently exposed to HA also showed enhanced resistance to inactivation by cholesterol. Thus, activated surfactant produces an optimal reduc-
tion of surface tension to values near or below 2 mN/m, not only during adsorption (both initial and postexpansion) but also during compression-expansion cycling (under continuous and fast dynamic regimes) despite the presence of serum or cholesterol, leading to full surfactant activity.

DISCUSSION

We demonstrated that HA strongly enhances the transfer of surfactant to the air-liquid interface and the resistance of surfactant to inactivation by serum or cholesterol. Systematic analysis shows that a threshold concentration close to the overlap concentration of HA is required to obtain these effects. Importantly, the beneficial effect of HA is retained even after removal of HA from the bulk solution.

The effect of polymeric HA and other polymers to reverse or prevent inactivation of pulmonary surfactant has so far been explained as a direct effect of depletion forces induced by the polymer on surfactant complexes, i.e. these physical forces have been proposed to push surfactant structures toward each other, leading to aggregation of surfactant complexes, and toward the interface, overcoming steric barriers such as those formed by serum proteins that occupy the interface and, thereby, recovering surfactant activity at the interface (8, 9). However, this model cannot explain several effects of HA observed in this study. First, it is difficult to understand how depletion forces would promote the reactivation of surfactant that was impaired by a different mechanism of inactivation, such as the membrane-perturbing effect of cholesterol (7). Second, the resistance to inactivation is retained after removal of HA from the surfactant even though depletion forces should disappear in this case (or at least be greatly diminished if minute amounts of HA remain in the sample).

The lack of interaction between surfactant complexes and hyaluronan molecules, evidenced from the QCM-D binding...
studies (Fig. 1) and from the HA-independent compression isotherms (Fig. 2), indicates that the effect of HA on surfactant activation is not related to specific biochemical interactions between HA and surfactant. To explain our observations, we propose that the effect of HA originates from physical phenomena (osmotic pressure and depletion forces) but that these phenomena act mostly indirectly, through the induction of irreversible structural and compositional changes, leading to a "primed" form of surfactant with better surface activity and the potential to overcome inactivation. The details of the proposed mechanisms of HA action are described in the following section.

Osmotic Pressure—The polymer meshwork offers a high resistance to the bulk flow of solvent. Thus, HA and other polymers prevent excessive fluid fluxes through tissue compartments. Furthermore, the osmotic pressure of HA solution is non-ideal, i.e. it increases non-linearly with the concentration at concentrations around and above the overlap concentration \( c_{ol} \). This relationship makes HA and other polysaccharides excellent osmotic pressure buffering substances because moderate changes in concentration lead to marked changes in osmotic pressure. Flow resistance together with osmotic buffering makes hyaluronan an ideal regulator of water homeostasis in the body (18).

The drastic effect of HA on the organization of surfactant membranes, as observed in our GUV models (Fig. 3), is reminiscent of results reported previously (6) that describe that another polymer, PEG, may also activate surfactant without entering into the aqueous spaces between bilayers. Native surfactant membranes have been shown to be fully permeable to water and polar solutes (42). Surfactant proteins form pores (43) and, thereby, facilitate the movement of water and small solutes. Polymers of sufficient size, on the other hand, are expected to be excluded from entering surfactant vesicles. The HA-promoted decrease in size of the GUVs (Fig. 3), therefore, may indicate that the difference in HA concentration between the inside and the outside of the vesicle is creating an osmotic pressure that induces the flow of water and small solutes toward the outside.

For polymers in good solvent, such as HA in saline solution (40), the osmotic pressure \( \Pi \) is known to increase with polymer concentration \( c \). Notably, the sensitivity of \( \Pi \) to changes in \( c \) is predicted to increase around the overlap concentration \( c_{ol} \). Although \( \Pi \) increases linearly with \( c \) below \( c_{ol} \), it increases supralinearly above \( c_{ol} \) (with a scaling up to \( c^{9/4} \) (44, 45)). The distinct increase in the sensitivity around \( c_{ol} \), which arises from the encounter of HA strands in the meshwork of interpenetrating polymers, may explain why the beneficial effect of HA sets in at this particular concentration.

The osmotically driven decrease of the vesicle volume would lead to membrane stress, and regions of the membrane that are particularly dynamic or prone to adopt a high curvature, such as domains enriched in unsaturated phospholipid species, could protrude and eventually be expelled from the vesicle to reduce the membrane surface. The HA-promoted selective exclusion of unsaturated phospholipids (Fig. 4) and cholesterol (Fig. 5) from surfactant MLVs supports this scenario. Selective exclusion of some components may, thus, play a key role in restoring the activity of surfactant when a membrane-perturbing molecule (such as cholesterol) is inactivating surfactant. In other words, there could be an irreversible HA-driven refinement of surfactant composition similar to the one that supposedly happens during compression-expansion cycling of interfacial surfactant films, known as "squeeze-out" (37, 46). It remains an open question whether HA, and perhaps other extracellular polymers, could also promote further structural and composi-
tional maturation/depuration of surfactant complexes in vivo when they are secreted by pneumocytes into the alveolar spaces. Pathological alterations of the properties of the extra-cellular alveolar medium could then additionally contribute to abnormal lung function and the impairment of surface activity.

Depletion Forces—Surfactant LUVs become aggregated when HA is present in the bulk phase, again at concentrations above 1 mg/ml (Fig. 6). The simplest explanation for this effect would be depletion forces, as already proposed earlier (6, 8, 9). Notably, the sensitivity of the depletion force to changes in polymer concentration $c$ is expected to increase around the overlap concentration $c_{\text{ol}}$ (47) and with $c^{3/2}$ above $c_{\text{ol}}$ (48) between two spherical objects. In analogy to the osmotic pressure, we suggest that this increase in sensitivity explains why the overlap concentration constitutes a threshold for efficient aggregation.

It remains to be elucidated whether the HA-promoted transformation of surfactant complexes involves exclusively a compositional depuration or whether it also leads to structural phase transitions that might be favored by the particular composition of surfactant. It has been proposed, for example, that the inverted H$_{II}$ phase promotes interfacial surfactant adsorption (49) and that hydrophobic surfactant proteins favor highly curved lipid organizations, such as cubic and H$_{II}$ phases (50, 51). Moreover, certain lipid systems enriched in phosphatidylethanolamine and cholesterol adopt non-lamellar phases under conditions of limited hydration (52). Therefore, it is conceivable that the local dehydration and compositional refinement

![FIGURE 8. Captive bubble surfactometry of native surfactant with or without transient exposure to HA in the presence of inactivating agents. A, surface tension after 5 min of initial adsorption (IA, black bars) or postexpansion adsorption (PEA, gray bars) of NS or native surfactant transiently exposed to 2.5 mg/ml HA in the absence or presence of inactivating agents (serum or cholesterol (Chol)). B, isotherms of the first, 10th, and 20th compression-expansion dynamic cycles of interfacial films formed by the different samples indicate restoration of normal surface activity after pre-exposure of surfactant to HA despite the presence of inactivating agents.](image-url)
promoted by HA may induce in surfactant membranes structural transitions toward non-lamellar phases, especially at the local environments where surfactant proteins are distributed. Those lipid-protein assemblies could be responsible for the higher ability of primed surfactant to transfer material into the interface. The fact that non-lamellar lipid organizations could mimic the structure of possible transient states during fusion of membranes with the interfacial film would support this hypothesis (50). Complementary experiments applying x-ray scattering or phosphorous NMR to HA-primed surfactant samples should provide important information to confirm the implication of possible non-lamellar phases in the ability of polymers like HA to promote surface activity.

It has been reported previously that the addition of HA increases the viscosity of surfactant preparations (4), thus imposing a physical barrier to the application of HA-surfactant mixtures in vivo. Moreover, introduction of polymeric HA into the airway may reduce clearance of liquid from the air spaces, and degradation may result in undesired low molecular weight, proinflammatory forms of HA (53–55). Unfortunately, the viscosity of hyaluronan solutions above the overlap concentration increases rapidly with concentration (18). This may be an insuperable problem because viscous solutions may not spread properly when introduced into the alveolar air spaces, making it difficult for surfactant to reach the distal alveoli. Our results show that elimination of HA from the mixture does not revert the effect of the polymer meshwork to enhance surfactant adsorption into the interface (Fig. 7). This means that, although entropic forces may act on the bulk phase, surfactant aggregation and/or compositional refinement are maintained when HA is eliminated, bringing surfactant to an activated state. That would mean that HA may not have to be present in surfactant preparations for their potential in vivo use. This would eliminate the viscosity problem and allow production of highly active surfactant preparations for therapeutic application under challenging conditions, such as those existing in an injured lung.

Finally, we have shown that HA-activated surfactant is able to efficiently cross the steric barrier imposed by a serum film adsorbed at the interface of an air bubble in the captive bubble surfactometer, confirming the substantially enhanced resistance to inactivation of HA-primed surfactant (Fig. 8). Activation of surfactant by HA not only provides to surfactant the ability to cross those inactivating steric barriers but also restores surfactant activity during compression-expansion cycling, resulting in a completely functional surfactant even in the presence of inactivating amounts of serum. In addition, HA priming of surfactant also prevents the deleterious effect of membrane-perturbing molecules, such as cholesterol, during compression-expansion conditions. Overcoming the deleterious effect of inactivation by different agents offers the possibility to develop efficient therapeutic strategies for the treatment of acute respiratory distress syndrome or meconium aspiration syndrome.

Further development of inactivation-resistant surfactants still requires assessing the ability of primed surfactant to work in vivo under the challenging conditions imposed by an injured lung and exploring to what extent activation by transient exposure to polymers, such as HA, produces a better outcome of the respiratory pathologies compared with common clinical surfactants currently in use.

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