Molecular and biophysical basis for the disruption of lung surfactant function by chemicals

Da Silva, Emilie; Autillo, Chiara; Hougaard, Karin Sørig; Baun, Anders; Cruz, Antonio; Perez-Gil, Jesus; Sørli, Jorid Birkeland

Published in:
Biochimica et Biophysica Acta. Biomembranes

Link to article, DOI:
10.1016/j.bbamem.2020.183499

Publication date:
2021

Document Version
Publisher's PDF, also known as Version of record

Citation (APA):
Molecular and biophysical basis for the disruption of lung surfactant function by chemicals

Emilie Da Silva, Chiara Autilio, Karin Sørig Hougaard, Anders Baun, Antonio Cruz, Jesus Perez-Gil, Jorid Birkelund Sørli

Department of Environmental Engineering, Technical University of Denmark, Kgs. Lyngby, Denmark
National Research Centre for the Working Environment, Copenhagen, Denmark
Department of Biochemistry and Molecular Biology, Research Institute “Hospital 12 de Octubre (imas12)”, Complutense University, Madrid, Spain

ARTICLE INFO

Keywords: Adverse outcome pathway Lung surfactant Constrained drop surfactometer Toxicity DPPC Pulmonary surfactant protein

ABSTRACT

With the intention to move away from animal testing for the toxicological evaluation of chemicals comes the need to develop new approach methodologies which are mechanism-anchored and target relevant key events leading to an adverse outcome. To date, no validated alternative methods are available for studying the acute inhalation toxicity potential of airborne chemicals but the constrained drop surfactometer measuring the surface tension of a drop of lung surfactant presents as a promising candidate. Indeed, the correlation of the increase in minimum surface tension of lung surfactant in vitro with changes in the breathing patterns of mice after inhalation of test compounds has been shown in multiple studies. However, the causal factors leading to lung surfactant inactivation remain speculative. This paper combines molecular and biophysical methods (constrained drop and captive bubble surfactometers, Langmuir-Blodgett balance, epifluorescence microscopy, cryogenic transmission electron microscopy, and differential scanning calorimetry) applied to purified porcine lung surfactant and dipalmitoylphosphatidylcholine interfacial films to gain insights into the disruption of lung surfactant function by three chemicals known to show acute inhalation toxicity (trimethoxyoctylsilane, methyl 3-oxo-2-pentylcyclopentaneacetate, and diisopentyl ether). The results of this study suggest that the test chemicals intercalate between the phospholipids at the air-liquid interface, reduce the stability of the films, and decrease the cohesivity of interface-associated multilayered structures thereby perturbing the lung surfactant surface activity. These findings contribute to a better understanding of chemically-induced lung surfactant function disruption.

1. Introduction

To move away from animal testing for the toxicological evaluation of chemicals, it is essential to develop new approach methodologies which are not only more cost-effective and less time consuming, but also and more importantly, physiologically relevant for humans [1,2]. In this context, the consideration of adverse outcome pathways (AOPs) is essential to develop specific assays, which target the key events linking a molecular initiating event to an adverse outcome [3–5]. Several AOPs addressing inhalation toxicity have been proposed [6]. Among those, the AOP 302 (https://aopwiki.org/aops/302) describes a pathway to acute inhalation toxicity initiated by the interaction of airborne compounds with the lung surfactant system [6]. In fact, lung surfactant constitutes the first barrier that airborne chemicals encounter in the alveolar region. This liquid layer coats the surface of the alveoli and consists of a mixture of 90% lipids (mostly phospholipids) and 10% proteins, including the lung specific surfactant-associated proteins (SP-A, SP-B, SP-C and SP-D) [7]. Lung surfactant is vital to maintain lung structure and function. It increases pulmonary compliance and prevents alveolar collapse by reducing the surface tension in the lungs. Impairment of lung surfactant function is potentially life-threatening. Therefore, the inhibition of lung surfactant function has been proposed as a predictor of chemically induced acute inhalation toxicity [8–10].

No validated alternatives to animal testing are currently available for acute inhalation toxicity, but the constrained drop surfactometer (CDS), addressing the disruption of lung surfactant function, presents as a good candidate [11]. The CDS measures the surface tension of a drop of lung surfactant before and during exposure: the inactivation of the lung
surfactant function is noted as an increase in the minimum surface tension.

In vivo, the inhibition of the lung surfactant function would lead to the collapse of the alveoli, thereby reducing the volume of air displaced during breathing (tidal volume) and perturbing respiration. The correlation of the increase in the minimum surface tension in the CDS in vitro with the decrease in tidal volume in a mouse model of inhalation in vivo was demonstrated after exposure to impregnation spray products [12], bile salts for inhaled drug formulation [13] and zinc oxide nanoparticles [14]. Further, correlation with cases of accidental intoxication in humans was established for some consumer products [12,15].

The objective of this paper was to provide mechanistic insights into the disruption of lung surfactant function by chemicals. A combination of molecular and biophysical assays was applied to study the structural and functional changes occurring after exposure of lung surfactant films to three chemicals relevant to human exposure and with documented clinical signs of toxicity in vivo following inhalation.

This study first describes the changes in native purified porcine lung surfactant (NS) function after exposure to trimethoxyoctylsilane (TTS), methyl 3-oxo-2-pentylcyclopentanecacetate (MOP) and diisopentyl ether (DPE) using the CDS and the captive bubble surfactometer (CBS). A combination of biophysical and structural methods was then applied to understand the perturbing effects of these compounds on the organization of interfacial films and membranes made of NS or 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), the main surface-active phospholipid in lung surfactant. Langmuir-Blodgett (LB) isotherms coupled with epifluorescence microscopy was used to investigate the molecular interaction of the selected chemicals with DPPC monolayers, while cryogenic transmission electron microscopy (cryo-TEM) and differential scanning calorimetry (DSC) were used to reveal changes in the molecular structure of surfactant membranes.

2. Material and methods

2.1. Materials

2.1.1. Lung surfactant models

Two surfactant models were used: native purified porcine lung surfactant (NS) and interfacial films made of DPPC, the main phospholipid in lung surfactant. Langmuir-Blodgett (LB) isotherms coupled with epifluorescence microscopy was used to investigate the molecular interaction of the selected chemicals with DPPC monolayers, while cryogenic transmission electron microscopy (cryo-TEM) and differential scanning calorimetry (DSC) were used to reveal changes in the molecular structure of surfactant membranes.

2.1.2. Chemicals

TTS (CAS 3069-40-7) is used as an adhesive and binding agent, for instance in the manufacture of machinery and vehicles and in the construction of buildings. Workers may be exposed through roller application and brushing. MOP (CAS 24851-98-7) is used for the formulation of fragranced items and as an intermediate for the manufacture of other chemicals. Workers and consumers are mainly exposed from cleaning products, air-care products, perfumes, and consumer end-use biocides. DPE (CAS 544-01-4) is used as an intermediate in the manufacture of chemicals and as a solvent in coating products for semiconductors. Inhalation exposure has been reported during production and manufacture.

Each chemical has previously been tested in vivo for acute inhalation toxicity according to the OECD test guidelines 403 and 436 for registration purposes. Following a 4-h inhalation exposure, the animals presented clinical signs of perturbed respiration: changes in the breathing frequency, shallow respiration and respiration sounds among others [17-19].

TTS, MOP and DPE were obtained from Sigma-Aldrich. The structure and relevant physico-chemical properties are summarized in Fig. 1 in Supplementary information and in Table 1 respectively. The relatively high vapor pressure of DPE at 273 Pa at 25 °C means that the compound was very easily volatilized in the assays. At the same temperature, the vapor pressures of TTS and MOP are only 0.2 Pa.

2.2. Intrinsic interfacial activity of the chemicals

The intrinsic interfacial activity of TTS, MOP and DPE was studied in the absence of phospholipids by monitoring the surface pressure over time in a Teflon cup (Nima Technology Limited) with 1.5 mL of sub-phase (bidistilled water) at constant surface area, continuously stirred at 25 °C. Pressure measurements were obtained using a Wilhelmy plate (Whatman filter paper 1441-070, dust free). Sufficient chemical was injected in the sub-phase to lead to a rise of the surface pressure. Measurements were obtained from two independent experiments, unless stated otherwise.

2.3. Constrained drop surfactometer

Inhibition of NS function was tested using an optimized version of the flow-through CDS [9,20]. A drop of NS (10 μL, 1 mg/mL) was placed on a hollow based pedestal with a sharp edge and cycled (20 cycles per minute) during the entire experiment by pushing the required volume of buffer in and out. After recording the baseline, NS was exposed for 60 s to the pure aerosolized compounds that were led from a glass syringe into a Pitt no.1 jet nebulizer [21] by means of an infusion pump (New England Medical Instruments Inc.). Images of the NS drop were continuously recorded by camera and analyzed by axisymmetric drop shape analysis (ADSA) software to measure surface tension as well as surface area [22]. The compressibility of NS was calculated as the slope of the line connecting the maximum area to the minimum area before and after exposure to the chemicals as previously defined [23]. For the baseline to fulfill the acceptance criteria, the minimum surface tension had to reach values below 5 mN/m with a reduction in relative surface area of less than 0.25. Only results from experiments that complied with these criteria were included in the analyses. The experiments were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Physico-chemical properties of trimethoxyoctylsilane (TTS), methyl 3-oxo-2-pentylcyclopentanecacetate (MOP) and diisopentyl ether (DPE) [17-19].</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTS</td>
<td>MOP</td>
</tr>
<tr>
<td>Molecular weight (g/mol)</td>
<td>234.41</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>-66</td>
</tr>
<tr>
<td>Boiling point (°C at 1013 hPa)</td>
<td>227</td>
</tr>
<tr>
<td>Vapor pressure (Pa) at 25 °C</td>
<td>0.2</td>
</tr>
<tr>
<td>Water solubility (g/L) at 20 °C</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Log Kow</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*In contact with water, TTS hydrolyses to (2,4,4-trimethylpentyl)silanetriol and ethanol. Kow: octanol-water partition coefficient.*
repeated five times for each chemical.

The exposure chamber was placed in a box heated to 37 °C and the pressurized air was heated prior to entering the jet nebulizer, to perform the experiments under physiologically relevant conditions. The temperature retrieved from the data logger (Tinytag Plus 2, TGP-4017, Gemini Data Loggers Ltd) placed close to the pedestal was 36.6 ± 0.6 °C for all experiments.

2.4. Captive bubble surfactometer

The biophysical properties of NS were also evaluated at 37 °C using the CBS [24,25] in the presence or absence of 10% (mol chemical/mol with respect to phospholipids, PL). The glass chamber contained 5 mM Tris-HCl pH 7, 150 mM NaCl and 10% w/w sucrose (to increase the buffer’s density and facilitate the surfactant’s injection against the floating bubble). A small air bubble (0.05 cm³) was formed and floated against a hydrophilic roof made of 1% agarose gel. Prior to testing, NS (10 mg/mL, 0.4 μL) was incubated with the tested chemical (diluted to 1/100 in DMSO to a nominal concentration of 39, 44, and 49 μmol/L for TTS, MOP and DPE respectively) in a Parafilm®-sealed glass tube. In 1 h at 37 °C with shaking every 10 min at 700 rpm. The sample was then injected close to the air-liquid interface of the bubble using a transparent capillary connected to a microsyringe. Changes in the shape of the bubble over time were recorded by camera, and the images were analyzed to derive the surface tension and area from the shape of the bubble [26]. NS adsorption into the air-liquid interface was measured by the rate of reduction in surface tension per second. After the initial adsorption, the chamber was sealed and the bubble was rapidly (1 s) expanded to a final volume of 0.15 cm³ for the study of post-expansion adsorption (minimum surface tension reached and adsorption rate). Five minutes after expansion, four quasi-static cycles were performed by altering the bubble volume by approximately 26% over 1 min in each direction (compression and expansion) with 1 min pause between successive cycles. Finally, the bubble was cycled at a frequency of 20 cycles per minute and 20 dynamic cycles were recorded. Hysteresis (area of the loop delimited by the expansion and compression curves) and compressibility (slope of the line connecting the points of maximum and minimum compression) were calculated (see Fig. 2 in Supplementary information for an illustration of the calculations) [47]. Three independent experiments were performed for each condition. An illustration of the operational scheme of captive bubble surfactometer experiments can be found in Hidalgo et al., 2017 [27].

2.5. Surface pressure – area isotherms and Langmuir-Blodgett films

The biophysical properties of DPPC interfacial films with and without TTS, MOP or DPE were assessed in a Langmuir—Blodgett trough (200 cm², Nima Technology Limited, Coventry, UK) filled with a bidistilled water subphase and equipped with a continuous Teflon ribbon barrier to avoid film leakage during compression [28]. DPPC (in chloroform-methanol 2:1) and the chemical (1/100 in chloroform-methanol 2:1) were vortexed for 30 min at room temperature at a final ratio of 10% (mol chemical/mol PL). The fluorescent probe BODIPY-PC (1% mol/mol PL, Molecular Probes) was added to the mixture for epifluorescence microscopy experiments. After spreading, the solvent was allowed to evaporate for 10 min to allow for material equilibration at the interface before compression at 25 cm²/min. The trough temperature was kept at 25 °C.

Changes in surface pressure were measured with a paper Wilhelmy plate (Whatman filter paper 1441–070, dust free) and plotted against the area occupied per phospholipid molecule (Å²/mol). The area per lipid molecule was calculated knowing the area of the interface (in cm²), DPPC molecular weight (M_DPPC in g/mol), the sample concentration (C_DPPC in μmol/L), the volume of DPPC applied at the interface (V_DPPC in mL) and the Avogadro’s constant (N₀) according to Eq. (1). We assumed that all deposited lipids were transferred and confined at the interface.

\[
\text{area per molecule} = \frac{\text{area} \times M_{\text{DPPC}} \times V_{\text{DPPC}} \times N_0}{C_{\text{DPPC}}} 
\]

2.6. Cryogenic transmission electron microscopy

NS was incubated with the chemical (diluted to 1/100 in DMSO) for 1 h at 37 °C with shaking for 10 min every 10 min at 1200 rpm in a Parafilm®-sealed glass tube. The final ratio was 10% (mol chemical/mol PL). The samples (10 μL PL/μL, 4 μL) were applied onto holey carbon grids R1.2/1.3 (Quantifoil®) after glow-discharge and immediately blotted and vitrified in liquid ethane cooled by liquid nitrogen using a FEI Vitrobot cryo-plunger. The grids were transferred onto the tip of a Gatan 626 liquid nitrogen cooling holder (Gatan) and imaged by a JEM 1230 electron microscope (Jeol) operated at 100 kV. Micrographs were taken at 40,000x nominal magnification under low dose conditions with a complementary metal oxide semiconductor (CMOS) TemCam-F416 camera (TVIPS GmbH).

2.7. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to characterize the thermotropic phase behavior of phospholipids in the selected membrane models (DPPC and NS) in the presence or absence of the chemicals [31]. Unless stated otherwise, the sample and the reference material (buffer) were degassed prior to being inserted into separate sealed stainless steel vessels in a micro-calorimeter (No. MC-2, MicroCal). The samples were consecutively heated to 60 °C and cooled to 15 °C for 15 cycles at a rate of 30 °C/h. Data from the eighth cycles were analyzed with the software Origin (Origin Labs) after baseline correction and concentration normalization. The enthality (area under the curve, kcal/mol K), the transition temperature (temperature at which the excess heat capacity is maximal, °C), and the cooperativity (the width at half height of the main calorimetric peak) were obtained from the thermograms of three independent experiments.

DPPC (in chloroform-methanol 2:1) was mixed with each chemical (in chloroform-methanol 2:1) at a ratio of 10% (mol chemical/mol PL). The suspensions were then dried under a nitrogen stream for 30 min and then under vacuum for 2 h to remove chloroform traces (Univap, Unipond). The dried films were hydrated with a buffer solution (5 mM Tris, 150 mM NaCl, pH 7.4) at 45 °C, i.e. above the melting temperature of DPPC (T_m = 41 °C), with shaking at 1200 rpm every 10 min for 2 h using a ThermoMixer (Eppendorf ThermoMixer®). For DPE, three additional independent experiments were performed, where the chemical was added to the multimellar vesicle suspension of pure DPPC after degassing to limit the volatilization of the chemical (Table 1). In experiments with NS, it was incubated with TTS and MOP (diluted to 1/100 in DMSO to a nominal concentration of 39 and 44 μmol/L respectively) for 1 h at 37 °C with shaking for 10 min every 10 min at 1200 rpm in a Parafilm®-sealed glass tube to a final ratio of 10% (mol chemical/mol PL). In the case of DPE (diluted to 1/100 in DMSO to a nominal concentration of 49 μmol/L), the chemical was added to the sample after degassing to limit its volatilization.

Langmuir-Blodgett films were prepared by transfer onto a glass coverslip during continuously varying surface pressure [29] for observation of lipid domains formation along the whole compression isotherm.

\[
\text{area per molecule} = \frac{\text{area} \times M_{\text{DPPC}} \times V_{\text{DPPC}} \times N_0}{C_{\text{DPPC}}} 
\]
3. Results

3.1. Intrinsic interfacial activity of the chemicals

Injecting 1 μL (3.9 μmol) of pure TTS and 5 μL (22 μmol) of pure MOP led to an important and permanent rise in the surface pressure, demonstrating the capacity of both chemicals to adsorb at the air-liquid interface. However, the injection of 5 μL (24.5 μmol) of pure DPE in the sub-phase led to an only transient increase in the surface pressure (light grey line in the right panel of Fig. 1). After about 5 min, the surface pressure decreased back to 0 mN/m. Injecting twice as much of DPE led to a 2-fold increase in the time to return to the baseline, suggesting a progressive volatilization of the chemical when it reached the air-liquid interface (Fig. 1 and Table 1 in Supplementary information).

3.2. Constrained drop surfactometer

The interaction of the aerosolized chemicals with NS at the air-liquid interface was investigated using the CDS (Fig. 2). The presence of TTS, MOP and DPE led to a rapid increase of the minimum surface tension of preformed NS interfacial films to values exceeding 10 mN/m (grey dotted line) within 15 s after initiation of exposure (t = 0 s). The increase was higher for TTS and MOP as only a transient rise was observed for DPE in 3 out of 5 experiments. This observation correlates well with the transitory increase in surface pressure of pure DPE adsorbing to the interface (Fig. 1). The study of the surface tension – area isotherms revealed a considerable increase of the compressibility of NS when exposed to each of the three chemicals (Fig. 2B, Fig. 2 in Supplementary information and Table 2 in Supplementary information): the same reduction in area led to a significantly lower decrease in surface tension.

3.3. Captive bubble surfactometer

Complementarily to the flow-through CDS, where NS was exposed to chemicals right at the air-liquid interface, the biophysical properties of NS pre-mixed with the chemicals were studied in the enclosed environment of the CDS (Fig. 3 and Table 2 in Supplementary information). Surface tension during initial adsorption, post-expansion adsorption, quasi-static cycles and compression-expansion dynamic cycling (first, tenth and twentieth cycles) was measured in the absence and presence of 50% (mol chemical/mol PL) after 1 h incubation at 37 °C. The increase in the surface pressure of pure DPE adsorbing to the interface (Fig. 1). The study of the surface tension – area isotherms showed a greater hysteresis than the following cycles (2.24 ± 0.53 in the first cycle versus 0.40 ± 0.20 in the fourth cycle) suggesting reorganization of material at the interface during the first compression. Dynamic cycles in the absence of chemicals were characterized by a low minimum surface tension reached with a relative area reduction of less than 0.13 ± 0.02 (low compressibility).

Only slight modifications in the biophysical properties of NS exposed to TTS and MOP were observed. Exposure to both chemicals slightly decreased the post-expansion adsorption rate and increased the maximum surface pressure during rapid dynamic cycling to a small extent. The presence of MOP (but not TTS) raised the reduction in area required to reach the minimal tension during dynamic cycling. In contrast, when NS was exposed to DPE, both the initial and post-expansion adsorption rates were remarkably reduced (Fig. 3A-B and Table 2 in Supplementary information). Moreover, the compressibility of NS during the four quasi-static cycles was significantly higher despite a decrease in relative area of 0.43 ± 0.08. During the dynamic cycles, the minimum and maximum surface tensions, as well as the compressibility, were increased. The hysteresis was constant at all cycles. We refer to Fig. 3 in the Supplementary information for the results of all three repeats of NS with DPE.

3.4. Surface pressure – area isotherms and Langmuir-Blodgett films

Surface pressure – area (π-A) isotherms of DPPC interfacial films (formed as monolayers) in the absence or presence of TTS, MOP or DPE (10%, mol chemical/mol PL) were recorded at 25 °C (Fig. 4). DPPC is the main surface active component in lung surfactant, thought to enrich the surfactant film at the alveolar spaces at the end of expiration, once compression promotes the squeeze-out of the less stable unsaturated phospholipids [32]. The lateral compression-driven transition of DPPC monolayers is very sensitive to the perturbation by trace amounts of any component. The phase transition from liquid-expanded (LE) to liquid-condensed (LC) phase of DPPC in the absence of chemicals was observed as a plateau of the surface pressure at 12.7 ± 0.2 mN/m. In the presence of TTS and MOP, the phase transition occurred at higher pressures: 13.5 ± 0.2 mN/m, and 14.6 ± 0.3 mN/m respectively. The plateau characteristic of the coexistence of the LE and LC phases in DPPC monolayers disappeared in the presence of MOP. In addition, the phase transition started at a lower area per molecule in the mixed monolayers (68.9 ± 5.9 Å²/mol, 52.7 ± 3.5 Å²/mol and 59.1 ± 4.5 Å²/mol, with TTS, MOP and DPE respectively) compared to DPPC alone (81.8 ± 9.6 Å²/mol). The need for more compression to reach higher surface pressures is likely due to a partial fluidization of the condensed states. The limiting area per molecule A₀ (at very high surface pressures) was calculated by extrapolating the straight line of the isotherm at LC phase to 0 mN/m. The values of A₀ for the mixed films with MOP and DPE (55–60 Å²/mol) were lower than that of DPPC alone (70–75 Å²/mol).

![Fig. 1. Pressure - time kinetics for trimethoxyoctylsilane (TTS), methyl 3-oxo-2-pentylocyclopentanecacetate (MOP) and diisopentyl ether (DPE). The chemicals were injected into the sub-phase and the surface pressure was recorded over time. Injecting twice as much of DPE led to a two-fold increase in the time to return to the baseline: 5 μL in light grey, 10 μL in grey and 20 μL in black.](image-url)
This reduction of the mean area per lipid molecule could be explained by an increase of the packing state of DPPC in the presence of the chemicals, or, more probably, to a partial solubilization of the DPPC molecules into the sub-phase due to a detergent-like effect of the chemicals. Moreover, the highest pressure reached by the mixed films was significantly lower in the presence of MOP than in the pure DPPC monolayers (59.0 ± 1.4 mN/m versus 68.9 ± 3.6 mN/m). The irregular behavior at the end of the isotherm of films made of the DPPC/TTS mixture indicated that the mixed film was less stable than the DPPC monolayers at the maximal compression. Lastly, the persistence of the effects of the chemicals on the π-A isotherms, even at high surface pressures, indicated that the chemicals were not completely excluded from the interface during compression.

These observations were confirmed by comparing the compression-driven phase transition of the DPPC monolayers with and without chemicals at different surface pressures under epifluorescence microscopy. Pure DPPC films exhibited a coexistence of LE and LC phases observable as dark packed domains excluding the fluorescent dye BODIPY-PC and surrounded by the LE phase containing the dye. The condensed domains in the DPPC monolayers featured the typical chiral lobed shape previously described for non-equilibrium states [28, 29]. However, in the presence of all three chemicals, the domains lost their shape as pressure increased, displaying a higher number of dendritic-like branches. This indicated a substantial reduction in line tension, possibly as a consequence of the accumulation of the compounds at the inter-phase boundaries. The reduced fraction of the surface occupied by condensed domains and the grey area surrounding the domains, particularly in the presence of MOP, at surface pressures of 17 mN/m further supported the hypothesis of an accumulation of the chemical excluded from the condensed DPPC domains at the boundaries with the LE phase, preventing DPPC molecules from reaching the LC phase. At high surface pressures (around 50 mN/m), the edges of the domains appeared blurry, likely as a consequence of an inefficient segregation of probe-containing and probe-excluding phases. Alternatively, we cannot discard the possibility that the chemicals cause distortion upon accumulation at the boundaries and a subsequent destabilization of the interfacial layer that could thus fluctuate out of focus. Overall, the significant perturbation of the compression-driven phase transition of DPPC was in agreement with the isotherms previously described and the nature of the interaction with the DPPC interfacial monolayer seemed chemical-specific.

3.5. Cryogenic transmission electron microscopy

Cryo-TEM analyses were performed to gain information on the morphological and structural changes at the single vesicle level following the exposure of NS to TTS, MOP or DPE (Fig. 5). In the absence of chemicals, NS vesicles appeared as radially symmetric structures with concentrically arranged layers. However, after exposure to TTS, and MOP, the vesicles were smaller and had fewer layers. In the presence of DPE, the vesicles were mostly unilamellar and several of them featured open ends and fragmented layers.
DSC thermograms of the DPPC suspensions exhibited two calorimetric peaks (Fig. 6A). The first peak was small and wide and occurred around 35.5 °C, corresponding to the well-established pre-transition from the gel (Lβ) to the tilted condensed (Lβ′) phase. The second peak, narrower and enclosing more enthalpy, appeared around 41.5 °C and corresponded to the main transition from ordered and relatively dehydrated states (Lβ′) to more disordered and loosely packed liquid-crystalline (Lα) configurations. All three chemicals abolished the pre-transition phase. In addition, TTS, and to a higher degree DPE when...
added after the sample degas, shifted the main transition to lower temperatures and broadened the calorimetric peak of the main transition.

A broad calorimetric peak was observed between 15 and 40 °C for NS membranes (Fig. 6B). Overall, TTS, MOP and DPE shifted the enthalpy and the transition temperature of NS to slightly lower values. Specifically, TTS led to a reduction in transition temperature from around 29.5 °C to 28.4 °C, and the enthalpy decreased from 2.5 kcal/mol to 1.6 kcal/mol in the presence of MOP.

4. Discussion

The present study was designed to provide mechanistic insights into the disruption of lung surfactant function by chemicals to support the use of the CDS for in vitro prediction of acute inhalation toxicity. Specifically, this paper showed that the increased minimum surface tension and compressibility of lung surfactant observed in the CDS after exposure to TTS, MOP or DPE was caused by molecular perturbations of the interfacial films. Epifluorescence-coupled LB microscopy, cryo-TEM and DSC revealed important structural changes in DPPC and NS membranes and interfacial films.

The degree and the nature of the perturbations were chemically specific. When NS was exposed to aerosols of the chemicals at the air-liquid interface (in the CDS), the alterations in the surface activity were more pronounced with TTS and MOP than with DPE (Fig. 2). Interestingly, when NS was mixed directly with the chemicals (in the CBS), DPE impaired the NS function the most (Fig. 3). The apparent discrepancy in effects between the CDS and the CBS correlates well with the physico-chemical properties of each substance: in the flow-through CDS, where the chemicals in the air phase went through the system...
with a high flow rate, the exposure of NS to the highly volatile DPE (vapor pressure of 273 Pa at 25 °C, Table 1 and Fig. 1) was most likely limited compared to the exposure of NS to the chemical in the airtight chamber of the CBS. This underlines the importance of including the mode of exposure when interpreting and comparing results for different test systems.

Three essential features of lung surfactant are pre-requisites for lung function. First, lung surfactant must adsorb rapidly at the air-liquid interface to reach equilibrium surface tensions around 22–25 mN/m. This is achieved by the protein-mediated transfer of phospholipids to the interface [33]. Second, the surface tension reached upon compression of the interfacial film must be very low and the film must be stable. Only films with an air-exposed layer rich in saturated phospholipids are rigid enough to sustain extreme compression without collapsing at 37 °C. This requires compression-driven depuration of the interface from the less active unsaturated phospholipids and neutral lipids according to the proposed squeeze-out model [32]. The excluded material typically constitutes complex surface-associated multilayered structures under the air-liquid interface. It has been shown that the combination of the two surfactant proteins SP-B and SP-C plays an important role in sustaining film stability, by maintaining the interfacial association of off-plane structures along recurrent compression-expansion cycles [34]. Third, lung surfactant must spread efficiently from the reservoirs upon expansion. SP-B and SP-C have been shown to be essential in facilitating the reversible transfer of lipids from the complex multilayered structures in the sub-phase back to the air-liquid interface [35]. SP-A, on the other hand, also present in NS, has been reported to cooperate in sustaining a complex structure of the surface-associated reservoir of surfactant, with large amounts of material maintained close to the interface, ready to replenish the films upon expansion [36]. Only a finely tuned organization and content in both saturated and unsaturated phospholipids as well as surfactant-associated proteins allows the simultaneous optimization of these three features [35].

In the current study, the ability of NS to reach very low tensions upon compression and the low compressibility of the film could not be maintained after exposure to the chemicals, suggesting at least partial fluidization of the condensed states. This could be attributed to the intercalation of the chemicals between the phospholipid molecules at the interface: a greater compression was necessary to depurate the interface from non-active molecules before the film becomes competent to reach sufficiently low surface tensions. This hypothesis is supported by the reduced area per phospholipid molecule observed at the LE-to-LC phase transition and at the highest pressure, and by the reduced fraction of the surface occupied by condensed phases. In agreement, the grey areas around the DPPC domains observed by epifluorescence were indicative of a less efficient packing and the partial exclusion of the fluorescent probe in the presence of the chemicals (Fig. 4). The compressed and packed ordered phases furthermore appeared to be less stable, as shown by the marked hysteresis during cycling of NS in both the CDS and the CBS, the decreased collapse pressure observed in the LB isotherms (Fig. 4), and the reduced transition temperature of DPPC bilayers (Fig. 6). The perturbations of the biophysical properties of NS observed after exposure to TTS, MOP and DPE somewhat resemble those observed previously for lung surfactant exposed to exacerbated amounts of cholesterol or serum [25,37,38].

The analysis by cryo-TEM suggests an even stronger effect of the chemicals on the three-dimensional structure of the surfactant film. The fewer lamellae in the vesicles exposed to the chemicals and the much smaller vesicles observed after incubation with TTS (Fig. 5) are indicative of a loss of cohesivity of the multilayered structures and are
consistent with the increased compressibility of the interfacial films and the loss of cooperativity observed by DSC. These findings suggest an intrinsic instability causing frequent ruptures and reorganization of the surfactant membranes. It is also possible that the perturbations induced by these chemicals alter the lipid-protein and protein-protein interactions mediated by surfactant-associated proteins. It has been described that SP-B promotes interfacial adsorption, formation of membrane-membrane interactions, and as a consequence, film stability [35,39,40]. In contrast, SP-C promotes the fragmentation of membrane vesicles and the destabilization of surfactant films, activities that are counteracted in the presence of SP-B [41,42]. Recent findings suggest that SP-B and SP-C form part of common complexes in surfactant assemblies and modulate each other [43,44]. The oligomeric structure of the collectin SP-A has been also proposed to be important for the maintenance of a highly cohesive multilayered structure of the surface film [36]. The perturbations in surfactant structure observed after exposure to the chemicals are compatible with a perturbation of lipid-protein and protein-protein interactions and the subsequent dysregulation of the membrane-modulating activities of surfactant proteins. If the chemicals perturb the interaction between SP-B and SP-C, the fragmentation and fusion activities of each proteins could be unmasked causing a progressive transformation of the surfactant functional structures. This hypothesis is consistent with that of Larsen et al. (2004) who partially restored the surface activity of a synthetic lung surfactant exposed to a siloxane by addition of SP-B [45].

The data obtained from the LB isotherms of DPPC films and the thermograms of DPPC suspensions must be interpreted with caution, as they constitute a simplistic model of the otherwise very complex surfactant system. Nevertheless, because of its abundance in the lung surfactant and its major role in surface activity, the use of DPPC as a model is relevant and informative of how different chemical compounds may alter the packing and properties of the DPPC enriched regions, representative of the compressed films of surfactant at the end of exhalation.

Overall, these findings have significant implications for the understanding of how airborne chemicals can interact with the lung surfactant and inactivate its function. This study supports the use of the constrained drop surfactometer to inform on lung surfactant function inhibition in vitro as a relevant key event in the biological pathway leading to acute inhalation toxicity.

Further research should be undertaken to establish the role of surfactant-associated proteins and to identify the driving chemical structures and physicochemical properties of disruptors of the lung surfactant function. We speculate that the hydrophobicity of the chemicals is a key element, since it can facilitate the intercalation between the phospholipid molecules. Other relevant features include the polarisability of the chemicals and their ability to form hydrogen bonds with the phospholipids, as it was suggested for three hydrophilic resin acids [46]. Also, the amphipathicity and the ability to aggregate and form micelles or clusters in surfactant membranes may promote the partial solubilization of key surfactant components or disrupt lipid-protein or protein-protein interactions that are crucial to maintain functional structures and optimal surface activity.

5. Conclusion

New approach methodologies anchored in the physiologically relevant key events leading to an adverse outcome for inhalation toxicity of chemicals are highly needed, but currently validated methods are lacking. Among several promising methods, the CDS, relying on the monitoring of lung surfactant function, presents as a good candidate. The combination of biophysical and structural methods revealed that the inactivation of lung surfactant function by TTS, MOP and DPE was related to (i) a partial fluidization of the condensed states by intercalation of the chemicals between phospholipids at the air-liquid interface, (ii) a loss of stability of the compressed phases, and (iii) a loss of cohesive stickiness of the multilayered structures. The molecular perturbations identified in this study support the functional changes observed after exposure of NS films to chemicals in the CDS.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank Rafael Nuñez-Ramírez for technical assistance for the cryogenic transmission electron microscopy experiments. Research in the laboratory of C.A., A.C. and J.P.-G. has been supported by grants of the Spanish Ministry of Science and Innovation (RTI2018-094564-B-I00) and the Regional Government of Madrid (P2018/NMT-4389). Emilie Da Silva is supported by The National Research Centre for the Working Environment, and the Technical University of Denmark, Department of Environmental Engineering.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2020.183499.

References
