Inhalation of waterproofing spray products has on several occasions caused lung damage, which in some cases was fatal. The present study aims to elucidate the mechanism of action of a nanofilm spray product, which has been shown to possess unusual toxic effects, including an extremely steep concentration-effect curve. The nanofilm product is intended for application on non-absorbing flooring materials and contains perfluorosiloxane as the active film-forming component. The toxicological effects and their underlying mechanisms of this product were studied using a mouse inhalation model, by in vitro techniques and by identification of the binding interaction. Inhalation of the aerosolized product gave rise to increased airway resistance in the mice, as evident from the decreased expiratory flow rate. The toxic effect of the waterproofing spray product included interaction with the pulmonary surfactants. More specifically, the active film-forming components in the spray product, perfluorinated siloxanes, inhibited the function of the lung surfactant due to non-covalent interaction with surfactant protein B, a component which is crucial for the stability and persistence of the lung surfactant film during respiration. The active film-forming component used in the present spray product is also found in several other products on the market. Hence, it may be expected that these products may have a toxicity similar to the waterproofing product studied here. Elucidation of the toxicological mechanism and identification of toxicological targets are important to perform rational and cost-effective toxicological studies. Thus, because the pulmonary surfactant system appears to be an important toxicological target for waterproofing spray products, study of surfactant inhibition could be included in toxicological assessment of this group of consumer products.

Key Words: Waterproofing spray product; lung toxicity; inhalation; pulmonary surfactant.

Waterproofing spray products constitute a group of consumer products used to make materials, including textiles, leather, carpets, wood, glass, concrete, masonry and stone waterproof, and dirt repellents. The products are intended both for industrial and domestic use, wherefore both private and professionals are exposed. During the past decades, inhalation of these products has caused several cases of severe intoxication (Burkhart et al., 1996; Vernez et al., 2006; Woo et al., 1983). Exposures have given rise to development of acute respiratory distress syndrome (Weibrecht and Rhyee, 2011), reactive airway dysfunction syndrome (Khalid et al., 2009), and acute lung injury (Hubbs et al., 1997). Laboratory studies have shown that inhalation of waterproofing spray containing fluorocarbon resin can cause pulmonary collapse in mice (Yamashita and Tanaka, 1995). It has been reported that only a partial dose-response relationship appears in humans after exposure to waterproofing spray products, suggesting that a pronounced inter-individual difference exists (Duch et al., 2014; Vernez et al., 2006).

Recently, a new generation of waterproofing spray products has been developed, the so-called nanofilm spray products (NFPs). A variety of NFPs for different materials are sold in, e.g., DIY stores and supermarkets, and thus both professionals and private consumers may be exposed to the products. When these nanofilm products are applied on a surface, the product forms a film in situ by chemical reaction with the surface. The nanofilm is very durable and is only a few nanometers in thickness. For a description of the nanofilm chemistry, see Norgaard et al. (2010). Like the more traditional products, the NFPs are used to achieve easy-to-clean surfaces because many impurities adhere poorly to hydrophobic surfaces (Quere, 2002). Some film products even create an ultrahydrophobic surface, i.e., the contact angle with water exceeds 150°, which improves the water-repellent properties.

One NFP containing hydrolysates and condensates of a perfluorinated silane caused lethal effects in mice upon short-term inhalation (Norgaard et al., 2010). The product has now been withdrawn from the market, but other products with very similar composition are still available. In mice, the dose-response relationship of the NFP was unusually steep. Thus, no effect was seen in the airways in mice inhaling a concentration of 16.1 mg/m³, whereas lethal effects were observed at 18.4 mg/m³ of the perfluorinated silane NFP (Norgaard et al., 2010). Furthermore, the level of protein in the broncho-alveolar lavage...
fluid, a robust marker of acute lung injury (reviewed in Matthay and Zimmerman, 2005), was elevated after exposure to NFP. Also, similar silane-based “Magic Nano sprays” gave rise to pulmonary toxicity in humans (Hahn et al., 2008), and the toxicity of the product was confirmed in rats (Pauluhn et al., 2008). Exposure to especially one of the products, glass and ceramic spray, induced lung inflammation, hemorrhages, edema, and focal septal thickening in the animals.

The mechanisms behind the unusual toxicological pattern of NFPs and other waterproofing spray products remain to be elucidated, but it appears that both the active film-forming substances as well as the composition of the solvents play a role in the toxicity of the product (Norgaard et al., 2014). This may also, at least partly, explain why outbreaks are often seen following a change of formulation of the product, i.e., qualitative or quantitative alterations of the solvent or the polymer (Vernez et al., 2006). Understanding the toxicological mechanisms of action including identification of toxicological target is important in relation to interpretation of in vivo data and a prerequisite to develop meaningful in vitro screening assays.

Here, we show that an important toxicological target for the NFP product studied is the pulmonary surfactant, which is a surface-active mixture of lipids and proteins found in the alveoli and terminal bronchioles. The pulmonary surfactant is a prerequisite for a normal lung function, and extensive neutralization of surfactant may lead to life-threatening conditions (Lopez-Rodriguez and Pérez Gil, 2014). The results presented here may be applied in the development of in vitro screening of waterproofing products for deterioration of pulmonary surfactant function and thus for acute pulmonary toxicity.

MATERIALS AND METHODS

Animals. Inbred male BALB/cA male mice aged 5–6 weeks, weight 24.4 g ± 1.9 were purchased from Taconic M&B (Ry, Denmark) and were housed in polypropylene cages (380 × 220 × 150 mm) with pinewood sawdust bedding (Lignocel S8, Brogaard, Denmark). Each cage, housing up to 10 mice, was furnished with bedding materials, gnaw sticks, and cardboard tubes. The photo-period was from 6 A.M. to 6 P.M., and the temperature and mean relative humidity in the animal room were 21°C ± 0.2 and 55% ± 5 (mean ± SD), respectively. Cages were sanitized twice weekly. Food (Altromin no. 1324, Altromin, Lage, Germany) and municipal tap water were available ad libitum. Treatment of the animals followed procedures approved by The Animal Experiment Inspectorate, Denmark (No. 2006/561-1123-C3).

Chemicals. The investigated nanofilm product (NFP) intended for coating of non-adsorbing flooring materials was obtained from NanoCover (Aalborg, Denmark). The NFP contains hydrolysates and condensates (siloxanes) of 1H,1H,2H,2H-perfluoroctyl triisopropoxysilane dissolved in 2-propanol (Norgaard et al., 2010). The 2-propanol (99.9%), dichloromethane (99.8%), dipalmitoylphosphatidyl choline (DPPC, 99%) and methanol (99.9%) were from Sigma-Aldrich Denmark, Brondby, Denmark. The exogenous lung surfactant preparation, HL10, was extracted from pig lungs (LeoPharma A/S, Ballerup, Denmark) and contains phospholipids, SP-B, SP-C, and cholesterol as described previously (Vermehren et al., 2006). The human SP-B1–25 (Protein Data Bank ID: 1DFW, amino acid sequence from N to C terminus: FPPIPLYCWLCRALKIQAMIPKGM, Mw 2928.2, purity: 75.2%), the fluorescein isothiocyanate (FITC) labeled SP-B1–25 (purity 92.5%) and the modified human non-palmitoylated SP-C peptide, SP-C33 (Almlen et al., 2010) (amino acid sequence from N to C terminus: IPSSPVHLKRLKLLLLALLLILLLGALLMGL, Mw = 3615.8 Da, purity: 71.5%) were synthesized by CASLO laboratory (Lyngby, Denmark) and provided as lyophilized trifluoroacetate salts. SP-B1–25 and SP-C33 are the active parts of SP-B and SP-C, respectively (Almlen et al., 2010; Hawgood et al., 1998; Walther et al., 2007). The lipophilic dye 1,1’dioctadecyl-3,3,3’-tetramethylindodicarbocyanine (DiD, purity ≥ 95% measured at 644 nm) was from Invitrogen, Molecular Probes (Eugene, OR).

Exposure of animals and assessment of effects in the respiratory tract. The NFP or control solvent were aerosolized by continuous infusion of the solutions from a glass syringe to a Pitt no. 1 jet nebulizer (Wong and Alarie, 1982) by means of an infusion pump (New England Medical Instruments Inc., Medway, MA). The exposure air-stream was subsequently led through a Vigreux column to ensure homogenous mixing and then directed into a 20 l exposure chamber of stainless steel with a hemispherical lid and bottom made of glass. The flow rate through the chamber was ~20 l/min resulting in an air exchange rate of ~1 min⁻¹.

Groups of mice (n = 10) were placed in body plethysmographs in the exposure chamber and exposed head-only for 15 min to laboratory air in order to obtain individual baseline levels. Then, mice were exposed for 60 min to 18.4 mg/m³ NFP or an equivalent concentration of 2-propanol (the solvent control group). The 18.4 mg/m³ was the Lowest-Observed-Adverse-Effect Level in mice (Norgaard et al., 2010) and is ~5 times higher than the concentration measured during a simulated workplace exposure scenario (Norgaard et al., 2009). The exposure period was followed by a 30-min recovery period in which the mice were exposed to laboratory air. To assess exposure-related effects, the respiratory parameters during exposure and recovery were compared with pre-exposure baseline levels, i.e., each mouse served as its own control. The Notocord Hem data acquisition software (Notocord Systems SA, Croissy-sur-Seine, France) was used to collect respiratory parameters. Respiratory parameters, data acquisition, and calculations have been described previously (Larsen et al., 2004). Briefly, the Notocord system calculates several respiratory parameters, includ-
ing breathing frequency (min⁻¹), tidal volume (ml), expiratory flow rate (ml/s) as well as specific markers of nose and lung irritation (time of break and time of pause, respectively). A reduction in the expiratory flow rate may be due to bronchoconstriction. Narrowing of the bronchi gives rise to an increased resistance that reduces the expiratory flow. Also, inhibition of the pulmonary surfactant system will lead to increased airway resistance, which may reduce the expiratory flow (Enhorning, 2001).

Capillary surfactometry. In the healthy lung, the walls of the terminal conducting airways are covered with a thin surfactant layer, which is important to maintain patency of the bronchioles. This is possible because the low surface tension counteracts the capillary forces driven by the narrow bronchioles. If the function of the pulmonary surfactant is inhibited, i.e., the surface tension increases, liquid may accumulate in and block the narrow bronchioles (Enhorning, 2001). To study this phenomenon, the capillary surfactometer (Calmia Medical Inc., Toronto, ON, Canada) was used. Like in the lung, the capillary tube is not of even width; it has a constricted part with a diameter of 0.25 mm, which reflects the most narrow part of the human airways (Enhorning, 2001). In this part, a surfactant sample of 0.5 µl can be deposited. Then, a constant airflow of 0.3 ml/min is led through the capillary. This will remove the surfactant sample from the narrow section, leading to an open capillary with low resistance. If the pulmonary surfactant properties are intact, the capillary will remain open as long as the airflow continues for 2 min. If, in contrast, the pulmonary surfactant is inhibited by, e.g., a chemical, the liquid surfactant will collapse and re-enter the narrow part of the capillary repeatedly leading to an increase in resistance. In the lungs, this is apparent as a decreased expiratory flow. In vitro, the percent of time with an open passage (low resistance) through the capillary during the 2-min sample period may be used to quantify the pulmonary surfactant function.

The NFP, containing approximately 99% 2-propanol, was diluted in 2-propanol to a total volume of 20 µl, i.e., the concentration of solvent was the same in all samples. The diluted NFP was added to 50 µl of HL10 resulting in a final HL10 concentration of 4 mg/ml. The surfactant samples were analyzed in the capillary surfactometer after 15 min of incubation at 37°C.

Langmuir isotherms. The pulmonary surfactant contains phospholipids and the surfactant-associated proteins (SP) B and C as the main surface active components (Perez-Gil, 2008). SP-B and SP-C are small hydrophobic proteins associated with the phospholipids. These proteins enhance interfacial adsorption of surface-active molecules into the air-liquid interface in the lung. This way, they contribute to mechanical stability of the interfacial film (Lopez-Rodriguez and Perez-Gil, 2014). Although phospholipids per se can reduce the surface tension of water, presence of SP-B and, to some degree, also SP-C, is necessary in order to achieve all of the dynamic properties of the complete lung surfactant, including the near-zero surface tension values that are observed in the compressed film. SP-B furthermore enhances the efficiency of surfactant by reducing the degree of film compression necessary in order to reach a very low surface tension (Lopez-Rodriguez and Perez-Gil, 2014). Two other surfactant proteins, SP-A and SP-D, are not considered to be involved in the reduction of the surface tension (Lopez-Rodriguez and Perez-Gil, 2014). To study how NFP may interfere with lung surfactant components, including SP-B and SP-C, a Langmuir technique (Nag et al., 1998) was applied and HL10 was used as model of pulmonary surfactant. The HL10 was deposited on a subphase of 157 ml carbonate buffer (150mM NaCl, 2mM CaCl₂, 0.2mM NaHCO₃, pH = 7.0) in a KSV Mini-trough 1 (KSV Instruments Ltd, Helsinki, Finland) with a surface area of 243 cm². The buffer was kept at 25.0 ± 0.1°C. A HL10 dispersion in carbonate buffer was spread on this subphase with a Hamilton syringe in an amount sufficient to reach a surface pressure of 20 mN/m before compression. The compression was initiated 20 min after spreading to allow the surfactant to adsorb at the interface. The barrier speed during the compression/expansion cycles was 20 mm/min. The surface pressure was measured by means of a Wilhelmy platinum plate (KSV Instruments Ltd). KSV software (KSV instruments Ltd) was used for data analyses. To study the effect of NFP on lung surfactant, dry HL10 powder (15 mg) was dissolved in 189 µl of NFP solution in 2-propanol. The components were mixed well by vigorous vortexing, the 2-propanol was evaporated, and the mixture was incubated for 1 h at 37°C. The dry HL10-NFP mixture was then dispersed in 10 ml carbonate buffer and stirred overnight before spreading the dispersion on the buffer subphase in an amount sufficient to reach a surface pressure of 20 mN/m before compression. As NFP inhibited the surfactant properties of HL-10, it was studied whether replacement of the surfactant proteins SP-B and SP-C could restore the surfactant properties. To do this, SP-B or SP-C peptides were dissolved in 2-propanol at 1 mg/ml and added to the HL10-NFP solution at a 2.5% weight ratio of HL10 for SP-B, and at a 7.5% weight ratio of HL10 for SP-C. For the mixtures of SP-B and SP-C, a 3.6% weight ratio of HL10 for SP-B and a 7.5% weight ratio of HL10 for SP-C were applied. The 2-propanol was evaporated completely, and the dry HL10-NFP-SP mixture was dispersed in carbonate buffer and stirred overnight before spreading the dispersion on the buffer subphase.

Histology. Immediately after the end of the inhalation experiment, lungs were fixed in situ by instillation of 4% (v/v) buffered paraformaldehyde via a polyethylene tube introduced into the trachea. The lungs were inflated to a pressure of 25 cm H₂O, and the size of the lungs was controlled through an opening in the pleural sack on both sides. After 5 min of fixation, the lungs were removed in toto and further fixed for at least 24 h using the same fixative.

For immunohistochemistry, the tissues were embedded in paraffin and cut into sections of ~10 µm. The samples were
were assessed by comparison with the solvent exposed controls.

The immunoreactions were visualized by 1 h incubation with biotinylated swine anti-rabbit immunoglobulins (code no. E 353, Dako, Denmark) diluted 1:100 as the third layer, and finally stained by means of 3,3-diaminobenzidine for 30 min. The sections were counterstained with hematoxylin. The degree of morphological changes in the lungs and the immunostainings were evaluated blindly.

Confocal microscopy. To study the effect of NFP on the interfacial localization of the SP-B1–25 peptide, a water-in-oil emulsion was prepared using DPPC as the emulsifier. Briefly, 67 μg (20 nmol) of SP-B1–25 peptide end-labeled with the fluorophore FITC was dissolved in 2-propanol. It was deposited in a test tube by evaporation of the solvent, and 10 mg (13.62 μmol) DPPC was added and dispersed in 2.1 ml chloroform with ultrasound using a Sonifier cell disruptor (Branson, Danbury, CT). The organic phase was stained with 9.5 μg DiD/ml. A volume of 30 μl NFP was added to a final amount of 3 μl NFP/mg DPPC. In the control experiment, the NFP was substituted with 30 μl 2-propanol. To this volume, 350 μl of carbonate buffer was added, and the mixture was sonicated until a thick, milky-white emulsion was obtained. Fluorescence microscopy was performed by using a Carl Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Piezosystem jena GmbH, Jena, Germany) equipped with an argon laser (458 and 488 nm) and a HeNe laser (543 nm) using the LSM 510 software.

Mass spectrometry. A two-phase system was prepared in a 1.5 ml glass vial by adding 0.5 ml dichloromethane to 0.5 ml of a 25 μg/ml aqueous solution of the SP-B1–25 peptide and, subsequently, 40 μl NFP was added to the organic phase. The vial was shaken vigorously for about 1 min and the emulsion was then allowed to separate at room temperature for about 20 min. Aliquots of both phases were diluted five times with methanol and infused directly into the electrospray ion source of a Bruker micrOTOF-Q (Bruker Daltonics, Bremen, Germany) at a flow rate of 5 μl/min. The capillary voltage was 4.5 kV in the positive mode and 3.2 kV in the negative mode. The nebulizer pressure was 1 bar, whereas the flow rate and temperature of the drying gas were 4 l/min and 190°C, respectively. An ESI calibration solution containing fluoroalkylphosphazines (Agilent tunemix, Agilent Technologies, Santa Clara, CA) was used for external mass calibration resulting in accuracies of ±5 mDa for abundant ions (≥10⁴ counts).

Statistics. The effects of the NFP on the expiratory flow rate were assessed by comparison with the solvent exposed controls. Statistical significance was accepted if no overlap occurred between the 95% confidence intervals of the solvent control group and the NFP exposure group (cf. Fig. 1). Data from the capillary surfactometer were analyzed by one-way ANOVA followed by the Dunnett method for comparison with a control using the Minitab ver. 15 software. p values < 0.05 were considered statistically significant.

RESULTS

Respiratory Effects Following Inhalation of NFP

Mice were exposed for 15 min to clean air to establish the normal (baseline) breathing pattern of each mouse. This baseline period was followed by exposure to the aerosolized NFP at a concentration of 18.4 mg/m³. Control mice were exposed to an equivalent concentration of 2-propanol, the solvent used in the NFP. Inhalation of the NFP aerosol reduced the expiratory flow rate, indicating an increased airway resistance (Fig. 1). The reduction occurred gradually over the 60-min exposure period, and a statistically significantly reduced flow rate compared with the control group was reached after approximately 10 min of exposure. The flow rate did not normalize within the 30-min post-exposure recovery period, where the animals were exposed to clean air.

Effects of NFP on the Pulmonary Surfactant System

To study effects of NFP on the pulmonary surfactant system, the capillary surfactometer bioassay was applied, cf. Materials and Method section. The pulmonary surfactant formulation HL10 (Vermehren et al., 2006) in water left the capillary open 98.7 ± 1.52% of the time (Fig. 2A). Addition of 20 μl 2-propanol to the HL-10 formulation reduced the % time open to 83.3 ± 8.9%, which means that the solvent itself has a minor inhibitory effect of the surfactant function. Replacing a part of the 2-propanol with NFP gave rise to a concentration-dependent decrease in % open, clearly showing an inhibitory effect of NFP on the pulmonary surfactant. The physiological consequences of this inhibition have been shown to be liquid blocking of the terminal airways, leading to increased airway resistance (Enhorning, 2001).

Mechanism of Pulmonary Surfactant Inhibition

The effect of NFP on the pulmonary surfactant was studied using the Langmuir technique. In this assay, the surfactant film is repeatedly expanded and compressed, and the assay was applied to investigate the properties of the pulmonary surfactant film under dynamic conditions. Addition of NFP to the HL10 surfactant film dramatically changed the shape of the pressure-area isotherm (Fig. 2B). The condensation isotherm of the native HL10 was characterized by a collapse plateau at approximately 70 mN/m. The addition of NFP to HL10 mainly affected the plateau part of the isotherm, resulting in the loss of the collapse plateau, which suggests a reduced mechanical strength of the
HL10 film. In addition, the expansion curve for HL10 was characterized by a fast decrease of the surface pressure to the initial surface pressure for HL10. However, in the presence of the NFP, the drop occurred in a different manner, suggesting that the film reorganized differently in the presence of NFP during the expansion phase. By adding synthetic SP-B peptide (SP-B1–25), mimicking the natural SP-B (Hawgood et al., 1998; Walther et al., 2007), the impaired film could partly be restored (Fig. 2B). In contrast, addition of SP-C peptide (SP-C33), a model peptide of the natural SP-C, did only change the initial part of the pressure-area isotherm, whereas no effect was seen at higher compression state compared with the HL-10 + NFP isotherm (Fig. 2B). This suggests that SP-C33 only to a minor degree affected the surfactant properties of the impaired HL10 film. Furthermore, the effect of a combination of SP-B1–25 and SP-C33 was similar to SP-B1–25 alone (Fig. 2B), indicating that SP-C33 did not potentiate the effect of SP-B1–25 in this set-up.

**NFP Interaction with SP-B In Vivo**

The interaction between SP-B and NFP observed in vitro was confirmed in vivo. Thus, lungs from NFP-exposed mice were fixed in formalin immediately after end of the inhalation study, and tissue sections were stained with SP-B-specific antibodies. Whereas the presence of native SP-B was apparent in the lungs of solvent-exposed control mice (Fig. 3A), no SP-B could be detected in the lungs of NFP-exposed mice (Fig. 3B), suggesting that the perfluorinated siloxanes physically or chemically modify one or more epitopes on SP-B essential for binding to the SP-B-specific antibodies. The SP-B depletion was most prominent in the bronchioles.

**Chemical and Physico-Chemical Investigations of NFP/SP-B Interaction**

The function of SP-B in surfactant films is to interact with the head-group of surfactant lipids including DPPC, and thereby improve the adsorption, spreading, and surface insertion of these lipids (Nag et al., 1999). Thus, a prerequisite for proper function of SP-B is its proximity to DPPC. An interaction between NFP and SP-B may change the physico-chemical properties of SP-B. To study whether this may be the case, the physical localization of the SP-B1–25 peptide was studied by confocal microscopy of water-in-chloroform emulsions using DPPC as emulsifier (Fig. 4). For this purpose, the SP-B1–25 was labeled with the fluorophore FITC, and the organic phase (red) was visualized by the addition of the lipophilic fluorophore DiD. The addition of FITC-labeled SP-B1–25 peptide did not affect the physical stability of the emulsion and the FITC-labeled SP-B1–25 peptide (green color) was clearly located at...
LUNG DAMAGE CAUSED BY NFP

FIG. 2. *In vitro* studies on inhibition of pulmonary surfactant function by NFP. (A) NFP was added to the pulmonary surfactant (HL10). After 15 min incubation at 37°C, the pulmonary surfactant function was analyzed by using a capillary surfactometer. The high patency (expressed as % time open) in the 2-propanol control group (0 μl NFP) was concentration-dependently reduced when 2-propanol was replaced by NFP. Mean and SEM of 3–6 replicates are shown. * denotes significant difference from the control, 20 μl 2-propanol (ANOVA, p < 0.001). (B) Pressure/area compression and expansion isotherms of the surfactant films consisting of HL10, HL10 + NFP, HL10 + NFP + SP-B1–25 peptide, HL10 + NFP + SP-C33 peptide, or HL10 + NFP + SP-B1–25 + SP-C33 peptide on carbonate buffer subphases. The addition of 189 μl NFP to 15 mg HL10 significantly reduced the collapse plateau at ~70 mN/m. Addition of SP-B to the NFP/HL10 mixture partly restored the surfactant properties (upper panel), whereas addition of SP-C (middle panel) had little effect. A combination of SP-B and SP-C (lower panel) showed a plateau similar to that of SP-B alone. The curves for SP-B are representative of three measurements, whereas the curves for SP-C and SP-B+SP-C represent one measurement.

the water/chloroform interface. Addition of NFP to the emulsion had a pronounced effect on the localization of the SP-B1–25 peptide. Thus, a part of the SP-B1–25 peptide relocated from the water/chloroform interface into the organic phase, which suggests that the SP-B1–25 is removed from its site of action.

To study the interaction between the perfluorosilanes and SP-B1–25 peptide at the molecular level, mixtures of these components in simple two-phase systems of dichloromethane and water were analyzed by mass spectrometry using direct infusion electrospray ionization. Figure 5A shows a representative mass spectrum of the water phase 20 min after addition of NFP and the SP-B1–25 peptide followed by a vigorous mixing of the phases. The ions around m/z 929.9 and 1151.5 are assigned as the +5 and +4 charge states of SP-B1–25 complexes with the tetrasisloxanes from the NFP, respectively. The insert shows the ions around m/z 929.9 in more detail; the indicated differences of 8.4 Th correspond to an isopropoxy group (C₃H₆O) with the mass 5e × 8.4 Th = 42 Da, consistent with the structures shown in Figure 5B. Thus, the peaks of m/z 913.1, 921.5, 929.9, and 938.4 are assigned as the SP-B1–25 complexes of the tetra-, tri-, di-, and mono-hydrolyzed tetrasisloxanes of 1H,1H,2H,2H-perflurooctyl trisopropoxysilane, respectively.

From the mass of the SP-B1–25/siloxane complexes, it can be concluded that the molecules do not react chemically, but interact due to physical adsorption. A covalent binding of the siloxanes to SP-B1–25 would imply a condensation reaction, which would lead to complexes with a lower molecular mass than those observed in the experiment. The formation of complexes seemed to occur only at the interface between the organic and the aqueous phases because no complexes were observed in one-phase systems containing NFP and the SP-B1–25 peptide, neither in dichloromethane nor in water.

**DISCUSSION**

The present study shows that inhalation of NFP aerosols reduces the expiratory flow rate, indicating an increase in airway resistance. This condition is observed in humans suffering from acute respiratory distress syndrome, which is associated with impairment of the pulmonary surfactant function (Matthay and Zemans, 2011). Pulmonary surfactant is located in the terminal bronchioles and in the alveoli and plays a key role for normal lung function. Pulmonary surfactant is composed of phospholipids and SP-A, -B, -C, and -D along with cholesterol (Perez-Gil, 2008). A major role of the SPs is to stabilize the surfactant film. SP-B is the only SP absolutely required for lung function and survival (Clark et al., 1995, 1997; Melton et al., 2003; Nogee, 2004; Weaver and Conkright, 2001), but deficiencies in SP-C may also lead to respiratory pathologies (Lopez-
FIG. 3. Interaction of NFP and SP-B investigated by histology. Lung tissue sections from solvent-exposed control mice (A) and mice exposed to NFP (B). Lungs were collected immediately after end of the 30-min recovery period. Sections were stained with an SP-B-specific antibody. Arrows indicate a positive (A) and a negative (B) staining with SP-B antibodies, respectively, indicating that SP-B in NFP-exposed mice was altered or absent. The sections are from one mouse per group representative of the five mice analyzed in each group.

Rodriguez and Perez-Gil, 2014). The pulmonary surfactant is located in the alveoli and in the terminal bronchioles, ensuring a free passage of air to and from the alveoli. However, when the surfactant is inhibited, a reduced airway patency is seen and surfactant will block this part of the airways leading to an increased airway resistance. Using capillary surfactometry (Enhorning, 2001), we were able to demonstrate that NFP inhibited the lung surfactant, which leads to liquid blocking of the terminal bronchioles, explaining the reduced expiratory flow rate in the animals. Also, our histological analyses showed that mice exposed to NFP had lower levels of native SP-B in the bronchioles. Exposure to higher levels (42.4 mg/m³) of the same NFP gave rise to alveolar collapse (atelectasis) and emphysema (Norgaard et al., 2010) showing that the alveoli also may be affected by these products.

The primary role of SP-B is to enhance the properties of surface active lipids in the pulmonary surfactant during respiration (Lopez-Rodriguez and Perez-Gil, 2014). At the end of expiration, the lung area is minimized, i.e., the surfactant film covering the alveoli is maximally compressed, whereby a high surface pressure (= low surface tension) is achieved, which counteracts alveolar collapse. In the presence of SP-B, the degree of surfactant film compression necessary to give a sufficiently high surface pressure is reduced (Lopez-Rodriguez and Perez-Gil, 2014). In the present study, the Langmuir isotherm (Fig. 2B) clearly showed that addition of NFP to pulmonary surfactant inhibits this function of SP-B as the reduction in area led to a lower increase in surface pressure compared with the pulmonary surfactant itself. Once the surfactant was inhibited by NFP, its properties could partly be restored by addition of novel SP-B1–25, as apparent from the steeper pressure-area isotherm. In contrast, addition of the SP-C33 peptide had little effect under the applied test conditions.

Chemical analyses demonstrated that the perfluorosilanes and SP-B1–25 peptide interacted non-covalently by physical adsorption and thus formed relatively stable complexes thereby inactivating the SP-B. It has previously been shown that perfluorinated compounds adsorb readily to proteins such as albumin (Bischel et al., 2010; Manus-Spencer et al., 2010), and the thyroid hormone transport protein transthyretin (Weiss et al., 2009). The affinity of perfluorinated compounds for proteins is most likely due to the polar hydrophobic nature of perfluorinated substances, as proposed by Biffinger and coworkers (Biffinger et al., 2004). It could be speculated that the perfluorosilanes with their hydrophobic perfluorcarbon tail in com-
FIG. 5. Non-covalent interaction between the SP-B$_{1-25}$ peptide and the perfluorosiloxanes in the NFP shown by mass spectrometry. The SP-B$_{1-25}$ peptide and the perfluorosiloxanes were allowed to react in a two-phase system of water and dichloromethane. (A) Electrospray ionization (+) spectrum of the water phase diluted five times with methanol. The +5 and +4 charge states of SP-B$_{1-25}$ complexes with tetrasiloxanes are observed around $m/z$ 929.9 and 1151.5, respectively. The ions around $m/z$ 1264.2 are the +4 charge states of SP-B$_{1-25}$ complexes with pentasiloxanes, whereas the +3 charge state of the SP-B$_{1-25}$ peptide can be observed around $m/z$ 976.9. The insert shows the ions around $m/z$ 929.9 in more detail. (B) Silane and siloxane structures: 1, non-hydrolyzed precursor silane; 2, fully hydrolyzed precursor silane; 3, tentative structure of the dihydrolyzed tetrasiloxane observed in a complex with SP-B$_{1-25}$ around $m/z$ 929.9 (cf. panel (A)).

Combination with hydrophilic silanol groups (Fig. 5B) somewhat mimic the structure of the phospholipids and facilitate both hydrophobic and hydrophilic interactions with proteins, as previously shown for perfluoroalkyl acids (Bischel et al., 2010).

The physico-chemical consequences of the interaction between NFP and the SP-B$_{1-25}$ peptide were studied using a water-in-chloroform two-phase system. This system has an interface that to some degree models the water/air interface of the lung. Whereas SP-B$_{1-25}$ in absence of NFP was located exclusively at the interface, addition of NFP led to distribution of SP-B$_{1-25}$ into the organic phase, i.e., SP-B$_{1-25}$ is removed from its site of action. Furthermore, binding or adsorption of perfluorosilane to the SP-B$_{1-25}$ peptide may hinder proper function of the peptide by the steric blocking of active sites of the molecule.

SP-B and SP-C are small hydrophobic proteins that are embedded in the phospholipid layer (Lopez-Rodriguez and Perez-Gil, 2014). Therefore, if a chemical should reach and potentially inhibit the function of SP-B, it has to be able to penetrate into the phospholipid layer. Only solvents with a certain degree of lipophilicity can do so, and this phenomenon may well explain why the toxicity of a waterproofing substance increased with increasing lipophilicity of the solvent (Norgaard et al., 2014).

New waterproofing and impregnation products are continuously being developed and marketed without being tested for inhalation toxicity, although exposures to these consumer products has given rise to several cases of intoxication each year. The toxicity of the products depends both on the active coating substance and the used solvents and minor changes in either of these components may have significant impact on the toxicity of the whole product (Norgaard et al., 2010, 2014). Consequently, there is a need for robust and reliable methods for investigating the acute lung toxicity of this type of consumer products. A mouse inhalation model may be useful for such screening. As the present study suggests that the pulmonary surfactant system is an important toxicological target for waterproofing spray products, surfactant inhibition assays should be developed to allow screening of these often quite complex mixtures of chemicals.

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