# Lung Damage in Mice after Inhalation of Nanofilm Spray Products: The Role of Perfluorination and Free Hydroxyl Groups

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Exposures to two commercial nanofilm spray products (NFPs), a floor sealant (NFP 1) and a coating product for tiles (NFP 2), were investigated for airway irritation, airway inflammation, and lung damage in a mouse inhalation model. The particle exposure was characterized by particle number, particle size distribution, and gravimetric analysis. BALB/cJ mice were exposed for 60 min to the aerosolized products at 3.3–60 mg/m$^3$ ($10^5$–$10^6$ fine particles/cm$^3$) measured in the breathing zone of the mice. Lung inflammation and lung damage were assessed by study of bronchoalveolar lavage fluid (BALF) cytology, protein in BALF, and histology. Mass spectral analysis showed that NFP 1 and NFP 2 contained hydrolysates and condensates of a perfluorosilane and alkylsilane, respectively. NFP 1 induced a concentration-dependent decrease of the tidal volume lasting for at least 1 day. Exposure concentrations above 16.1 mg/m$^3$ ($2.1 \times 10^6$ fine particles/cm$^3$) gave rise to significant increases of protein level in BALF and reduced body weight, and histological examination showed atelectasis, emphysema, and hemorrhages. A narrow interval between the no-effect level (16.1 mg/m$^3$) and the lethal concentrations (18.4 mg/m$^3$) was observed. The alkylsilane-based product (NFP 2) had no effect at the concentrations studied. Experiments with different types of perfluorinated silanes and alkylsiloxanes showed that the toxic effects did not arise solely from the perfluorination. The number of free hydroxyl groups in the silanes/alkylsiloxanes was also critical for the toxicity.

*Key Words:* nanoparticles; silane; alkylsiloxane; perfluorination.

Nanofilm spray products (NFPs) are a relatively new type of surface coatings for industrial and domestic use. Most of the NFPs induce nonstick properties when applied to surfaces. The NFPs are available for a wide range of surfaces, e.g., bathroom tiles, floors, textiles, and windows. The NFPs are sprayed onto a surface, and a thin (nano) film is formed by self-organization during evaporation of the solvent. A literature search in various patent databases yielded some information about the production of coating compositions that induce similar properties to a treated surface as the ones mentioned for the NFPs above (Brueck *et al.*, 2003; Fries *et al.*, 2001; Gross *et al.*, 2003; Schmidt *et al.*, 1997). These patents indicate that NFPs may be based on organo-functionalized silanes (e.g., fluorinated) and, in some cases, nanoparticles of for example silica or titantia. These silanes are "activated" through controlled hydrolysis and condensation reactions, which enable the formation of a self-assembling film during evaporation of the solvent, thus inducing the desired properties to the treated surface (Brinker, 2004; Hench and West, 1990; Schmidt, 2006; Sepeur, 2008). The result is an interconnected rigid network of functionalized organosiloxanes. The process is similar to the preparation of silica sols by polymerization of silicon alkoxides by addition of water and an acid catalyst (Hench and West, 1990).

The identity and concentration of all chemicals in the commercial products are seldom available from the product information, e.g., materials safety data sheets (MSDS). Consequently, the current knowledge of the chemical composition of the products is limited and insufficient for risk assessment. Recently, it was concluded on the basis of 102 cases with waterproofing spray products that "no threshold could be found to define a safe level of exposure" (Vernez *et al.*, 2006). Furthermore, it was concluded that improvement of the occupational and environmental conditions provided insufficient prevention of future outbreaks of waterproofing spray toxicity because the cause-effect relationship is unknown.

A number of cases have been reported worldwide after application of spray products to leather and textiles (Burkhart *et al.*, 1996; Heinzer *et al.*, 2004; Laliberté *et al.*, 1995; Lazor-Blanchet *et al.*, 2004; Okonek *et al.*, 1983; Vernez *et al.*, 2006; Woo *et al.*, 1983). The symptoms were dyspnea, breast pain, coughing, headache, and fever; some may have a severe reaction, e.g., lung inflammation with pulmonary edema and hemorrhage and reduced alveolar oxygen uptake resulting in reduced blood oxygen tension. Clusters of cases have been observed after reformulation of products, e.g., substitution of

one fluorinated polymer with another (cf. Hubbs *et al.*, 1997; Yamashita and Tanaka, 1995). It has been argued that the common solvents in the product and particles *per se* were unlikely causative agents of the reported symptoms (Vernez *et al.*, 2006; Yamashita and Tanaka, 1995). Recently, at least 153 severe cases with rapidly developing reactions have been registered by the Federal Institute for Risk Assessment in Germany. The individuals used a so-called Magic Nano spray product for coating glass and ceramic tiles (Pauluhn *et al.*, 2008). Major symptoms were vigorous cough and dyspnea. In some cases, lung edema was also confirmed by exposure of rodents to the same product (Pauluhn *et al.*, 2008). The causative agents, however, have not been identified.

It has been postulated that perfluorinated silanes may alter the surface properties of the lung lining fluid, e.g., by increasing the surface tension of the lung lining fluid (Yamashita and Tanaka, 1995). This could counteract the effects of lung surfactants resulting in alveolar collapse with the consequence of a cascade of potential lung-damaging effects (Vernez *et al.*, 2006; Yamashita and Tanaka, 1995). This is partly supported by the fact that the perfluorinated polymers appear to be potentially more toxic than non-fluorinated alkylsiloxanes (Yamashita and Tanaka, 1995).

Identification of common molecular structures of toxic substances in NFPs may allow substitution with less toxic compounds and thus prevent future outbreaks. Additionally, significant exposure to both surface-active chemicals and very fine particles formed in the air during application is possible during the use of these products (Nørgaard *et al.*, 2009). Taken together, these observations prompted us to investigate two common commercial NFPs for sealing surfaces: one with and one without perfluorinated silanes/alkylsiloxanes. This was carried out by the use of a mouse inhalation model that allows assessment of effects in the upper and lower airways (e.g., Kuwabara *et al.*, 2007). Toxic effects were evaluated by analysis of pulmonary function, inflammatory cells in bronchoalveolar lavage fluids (BALFs), and histopathology. The particle exposure was monitored, and the products were subject to detailed chemical characterization to enable identification of the potential causative agents.

## MATERIALS AND METHODS

*Animals.* Inbred male BALB/cA mice aged 5–6 weeks, weight 24.1 ± 1.7 g, were purchased from Taconic M&B (Ry, Denmark) and were housed in polypropylene cages (380 × 220 × 150 mm) with pinewood sawdust bedding (Lignocel S8; Brogaarden, Denmark). The cages, housing up to 10 mice each, were furnished with bedding materials, gnaw sticks, and cardboard tubes. The photoperiod was from 6:00 A.M. to 6:00 P.M., and the temperature and mean relative humidity in the animal room were 21°C ± 0.2°C and 55 ± 5% (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.* Ethanol (99.9%), 2-propanol (99.9%), formic acid (98–100%), 1H,1H,2H,2H-perfluorooctyl triethoxysilane (98%), and dodecyl trimethoxysilane (95%) were obtained from Sigma-Aldrich (Brøndby, Denmark). Bis-(1H,1H,2H,2H-perfluorooctyl) tetramethyldisiloxane (95%) was obtained from Apollo Scientific (Cheshire, UK). Denatured ethanol (93%) was purchased from Borup Kemi (Borup, Denmark).

*Nanofilm products.* Two types of NFPs were investigated in this study: one for coating of nonabsorbing floor materials (NFP 1) and one for coating of ceramic tiles (NFP 2). Both products are delivered in pump spray bottles. According to the MSDS supplied by the distributor, NFP 1 contains 2-propanol (solvent) and unspecified fluorosilane and NFP 2 contains ethanol and methanol (solvents) and unspecified alkylsilane. The coating of surfaces requires 10–40 g/m$^2$ depending on product type.

Chemical analysis using electrospray ionization mass spectrometry (ESI-MS) showed that NFP 1 and NFP 2 contained hydrolysates and condensates of 1H,1H,2H,2H-perfluorooctyl triisopropoxysilane and hexadecyl trimethoxysilane, respectively, see Figure 1. When applied to a surface, OH-groups will condense with each other forming a polysiloxane network. Excess silane and alkylsiloxane are wiped from the treated surface afterward, leaving a thin hydrophobic layer. Further details of the chemical analysis will be published elsewhere (Nørgaard, Wolkof, and Lauritsen, in preparation). Gas chromatography-mass spectrometry analysis of the emitted volatile organic compounds during spraying showed chloroacetones and 1H,1H,2H,2H-perfluorooctyl triisopropoxysilane from NFP 1 and several small aliphatic ketones from NFP 2 (Nørgaard *et al.*, 2009).

The concentrations of silanes in NFP 1 and NFP 2 were estimated in the following way: 1 ml NFP was transferred to a 2-ml glass vial and purged at ambient temperature to dryness by a gentle stream of nitrogen (purity 5.0, i.e. >99.999%). The mass of the nonvolatile fraction was determined gravimetrically in quadruplicate. NFP 1 and NFP 2 contained 1.1 ± 0.1% and 1.5 ± 0.1% silane by weight, respectively.

*Preparation of synthetic floor sealant, NFP 1.* To verify the effects observed for NFP 1, a synthetic NFP 1 was prepared from 1H,1H,2H,2H-perfluorooctyl triethoxysilane. In addition, this permitted an evaluation of the role of free hydroxyl groups for the toxicity of perfluorinated silanes/alkylsiloxanes; 1.5, 2.25, or 3.0 mmol of water (0.5, 0.75, and 1.0 mol water added for each hydrolyzable group, respectively) and 0.3 mmol formic acid was added to 1.0 mmol of 1H,1H,2H,2H-perfluorooctyl triethoxysilane in a 2.5-ml plastic vial. Furthermore, 100 μl of ethanol was added before the mixtures were shaken gently for 30 min and then allowed to stand at room temperature for 2 days resulting in clear homogenous solutions. The mixtures were then diluted with ethanol until 1.2% solutions were obtained. The solutions were stored in Teflon flasks at room temperature until use. Samples for ESI-MS experiments were taken to verify the degree of condensation/hydrolysis.

A clear tendency could be observed by adding 1.5, 2.25, and 3 mmol of water, respectively, where the degree of hydrolysis increased with the addition of water, thus leading to more free hydroxyl groups.

*Generation of test atmospheres and particle characterization.* The inlet flow rates of the NFPs used in the exposures are shown in Table 1. The mice were exposed to NFP 1, NFP 2, synthesized NFP 1, and solutions (1.2%) of 1H,1H,2H,2H-perfluorooctyl triethoxysilane (perfluorosilane) and bis(1H,1H,2H,2H-perfluorooctyl) tetramethyldisiloxane (perfluorodisiloxane) in ethanol, see Figure 1. The NFPs, chemicals, and solvent controls were aerosolized by continuously injecting 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 airstream was subsequently led through a Vigreaux column to ensure homogenous mixing and directed into a 20-l exposure chamber of stainless steel with a hemispherical lid and bottom made of glass resulting in an air exchange rate of 1.5 min$^{-1}$ (29.6 l/min).

Outlet air was passed through a series of particle and active coal filters before exhaust to the atmosphere. 2-Propanol and denatured ethanol were used as solvent controls for NFP 1 and NFP 2, respectively.
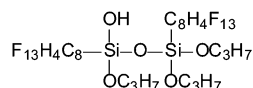
The particle exposure was measured by the use of four different types of particle measurements: The size distribution of nanoparticles was measured using a Fast Mobility Particle Sizer (FMPS; TSI Model 3091; Shoreview, MN),
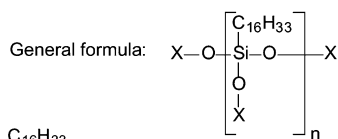
## NFP 1

General formula:
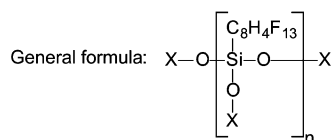


X = H, $C_3H_7$ or $C_8H_4F_{13}O_2Si$-Y where Y = H or $C_3H_7$
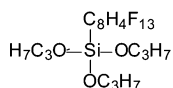
1H,1H,2H,2H-perfluorooctyl triisopropoxysilane

mono hydrolysed disiloxane
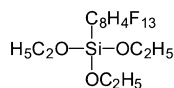
## NFP 2

General formula:



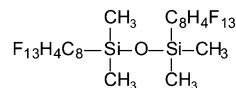X = H, $C_2H_5$ or $C_{16}H_{33}O_2Si$-Y where Y = H or $C_2H_5$
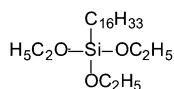
Hexadecyl triethoxysilane
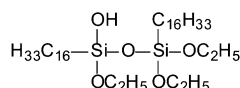
mono hydrolysed disiloxane

## Perfluorosilane and perfluorodisiloxane

1H,1H,2H,2H-perfluorooctyl triethoxysilane

Bis(1H,1H,2H,2H-perfluorooctyl) tetramethyldisiloxane

**FIG. 1.** Silanes and alkylsiloxanes of 1H,1H,2H,2H-perfluorooctyl triisopropoxysilane (NFP 1) and hexadecyl triethoxysilane (NFP 2). A nonhydrolyzed silane and a monohydrolyzed disiloxane are shown for each of the two NFPs.

which measures the electrical mobility particle size ($D_m$) in 32 channels with midpoints ranging from 6.04 to 523.3 nm.

The FMPS was operated at room temperature, and sampling was conducted through the standard FMPS cyclone Model 1031083 ($d_{50} = 1$ μm). Coarser particles were measured using an Aerodynamic Particle Sizer (APS; TSI Model 3321), which measures the aerodynamic particle size ($D_a$) of particles in 51 diameter classes with midpoints ranging from 0.542 to 18.4 μm. A Condensation Particle Counter (CPC; Grimm Model 5.403; Douglasville, GA) was used for the measurement of the total number of fine particles (4.5 nm [$D_{50}$, verified with tungsten oxide] to >3 μm). Particles were sampled at

## TABLE 1
Particle Concentration Data for Exposure Tests of (a) Floor Sealant (NFP 1) and (b) Tile Coating (NFP 2). Concentrations in Bold Are Used for *In Vivo* Exposure; Concentrations Marked With Asterisk Are Calculated. See the "Materials and Methods" Section for Details

| Inlet flow rate (ml/min) | Particle concentration (n/cm³) | Mass concentration in exposure chamber (mg/m³) | | | |
|---|---|---|---|---|---|
| | CPC | FMPS | APS | FMPS + APS | Filter |
| (a) | | | | | |
| 0.01 | 1.4E+05 ± 1.0E+04 | 0.1 ± <0.1 | 0.4 ± <0.1 | 0.5 ± 0.1 | 0.5* |
| **0.05** | **6.8E+05 ± 6.1E+04** | **1.4 ± <0.1** | **2.6 ± <0.1** | **4.0 ± 0.1** | **3.3 ± 0.7** |
| 0.075 | 1.0E+06 ± 7.2E+04 | 2.2 ± 0.1 | 3.8 ± 0.2 | 6.0 ± 0.3 | 5.9* |
| 0.1 | 1.5E+06 ± 1.1E+05 | 3.2 ± 0.1 | 4.9 ± 0.2 | 8.1 ± 0.3 | 8.5 ± 2.4 |
| **0.2** | **2.5E+06 ± 2.0E+05** | **6.7 ± 0.2** | **9.9*** | **16.6*** | **15.7 ± 1.2** |
| **0.21** | **2.1E+06*** | **10.1 ± 0.2** | **10.4*** | **20.5*** | **16.1*** |
| **0.23** | **2.3E+06*** | **10.2*** | **11.3*** | **21.5*** | **18.4*** |
| **0.3** | **2.5E+06*** | **13.3*** | **14.7*** | **28.0*** | **24.4*** |
| **0.5** | **4.6E+06*** | **22.1*** | **24.7*** | **46.8*** | **42.4 ± 1.1** |
| (b) | | | | | |
| 0.01 | 1.7E+05 ± 1.2E+04 | 0.2 ± <0.1 | 0.7 ± 0.1 | 0.9 ± 0.1 | 1.1* |
| 0.05 | 5.4E+05 ± 3.9E+04 | 1.3 ± <0.1 | 3.3 ± 0.3 | 4.6 ± 0.3 | 3.3 ± 0.1 |
| 0.075 | 8.5E+05 ± 6.1E+04 | 2.2 ± <0.1 | 4.4 ± 0.5 | 6.6 ± 0.5 | 9.4* |
| **0.2** | **1.9E+06*** | **5.5*** | **12.3*** | **17.8*** | **33.2 ± 7.3** |
| **0.5** | **3.1E+06 ± 2.2E+05** | **14.9*** | **30.7*** | **45.6*** | **60.0 ± 2.5** |
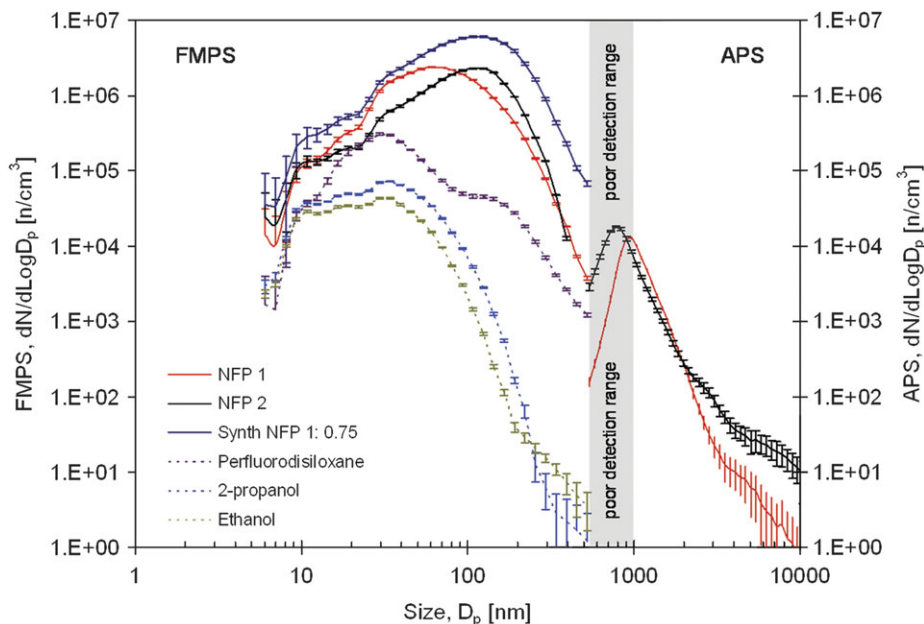
**FIG. 2.** Particle number size distribution spectra of NFP 1 and 2, synth NFP 1:0.75, perfluorodisiloxane, and the two solvents (2-propanol and ethanol) used in NFP 1 and NFP 2. The spectra of NFP 1 and NFP 2 were measured using both the FMPS and the APS. All other spectra were only measured using the FMPS. All data are presented at an inlet flow of 0.05 ml/min. Vertical bars indicate SDs.

positions in the center of the exposure chamber by the use of 1/8" Teflon tubes (length 10–15 cm) and 1/4" conducting flexible tube (~30 cm). All instruments were operated simultaneously at a sampling frequency of 1 s, and the sample flows were 10, 5, and 0.3 l/min for the FMPS, APS, and CPC, respectively. Theoretical mass concentrations were calculated on the basis of the FMPS and APS measurements assuming spherical particles and densities of 1H,1H,2H,2H-perfluorooctyltriethoxysiloxane (1.34 g/cm$^3$) and hexadecyl trimethoxysilane (0.89 g/cm$^3$) for NFP 1 and NFP 2, respectively (Table 1).

Finally, the mass concentration of the particle exposure was quantified by filter sampling using Millipore Teflon with a pore size of 0.45 μm (Millipore Co., Billerica, MA) at an air flow of 2 l/min for 2–36 min. Differential weighing of the filters was carried out on a Sartorius Microscale Model M3P (Sartorius GmbH, Göttingen, Denmark) after 24 h of acclimatization (humidity 50 ± 2.5%, 20°C ± 0.2) before and after filter exposure.

***Collection of respiratory parameters.*** The Notocord Hem (Notocord Systems SA, Croissy-sur-Seine, France) data acquisition software was used to collect respiratory parameters. For each experiment, up to 10 mice were placed in body plethysmographs in the exposure chamber, and animals were exposed head-only (Vijayaraghavan *et al.*, 1994). The acquisition program calculates *inter alia* the respiratory frequency (breaths/minute), time of inspiration (milliseconds), time of expiration (milliseconds), time from end of inspiration until the beginning of expiration, termed time of brake (milliseconds), time from end of expiration until beginning of the next inspiration, termed time of pause (milliseconds), tidal volume (VT, milliliters), and midexpiratory flow rate (milliliters/second). Comprehensive descriptions of the breathing parameters have been made elsewhere (Alarie, 1998; Larsen *et al.*, 2000; Nielsen *et al.*, 1999; Vijayaraghavan *et al.*, 1994). Data acquisition and calculations were performed as described previously (Larsen *et al.*, 2004).

***Acute airway irritation.*** To assess the airway irritation effects of the two NFPs, groups of mice (n = 7–19) were exposed for 60 min to concentrations of NFP 1 ranging from 3.3 to 42.4 mg/m$^3$ and NFP 2 from 33.2 to 60 mg/m$^3$, respectively, see Table 1. The 60-min exposure period was followed by a 30-min recovery period in which the mice were exposed to laboratory air.

Prior to exposure, a 15-min baseline period was recorded for each mouse. To assess the exposure-related effects, the respiratory parameters during exposure were compared with baseline levels, i.e., each mouse served as its own control.

The reversibility of the effects on the respiratory tract was assessed by collection of the respiratory parameters 22–24 h after cessation of the exposure to the NFPs.

***Bronchoalveolar lavage procedure.*** After collection of postexposure respiratory parameters, bronchoalveolar lavage (BAL) was performed by flushing the lungs four times with 0.8 ml of saline (0.9%) using a tracheal cannula. The recovered BALFs were pooled and centrifuged (500 × g, 10 min, 4°C). The supernatant was discharged, and the pellet was resuspended in a 200-μl phosphate-buffered saline containing heparin (20 IE/ml) and serum albumin (0.003%). The total number of cells was determined using a hemocytometer (Merck Eurolab). For differential counts, cytospin preparations were made (Cytospin2; StatSpin Inc., Norwood, MA) (55 × g, 4 min, room temperature). Slides were stained with May-Grünwald/Giemsa, and all slides were inspected by the same technician. Cells were identified by standard morphology and differentiated into neutrophils, eosinophils, epithelial cells, lymphocytes, and macrophages. For each slide, 200 cells were differentiated and the total cell count of each cell type was calculated.

The BALF supernatants were analyzed for content of protein by means of the bicinchoninic acid Protein Assay Kit. The operating procedure provided by the manufacturer (Pierce, Rockford, IL) was followed.

***Histology.*** In separate groups of mice (n = 9–10 per group), the lungs were fixed *in situ*, without preceding BAL procedure. The chests of the mice were opened and a polyethylene tube introduced into the trachea. The polyethylene tube was connected to a syringe containing 4% buffered paraformaldehyde, and the lungs were inflated with the fixative to normal size. After 5 min, the lungs were removed *in toto* and further fixated for at least 24 h. Tissues were embedded in paraffin in a standardized way (horizontal cut through the hilum regions), and subsequently, 7 μm–thick slices were cut and stained with periodic acid-schiff hematoxylin. The degree of inflammation and morphological changes in the lungs were evaluated blindly and independently by two researchers. In case of discrepancy, the slide was reassessed.
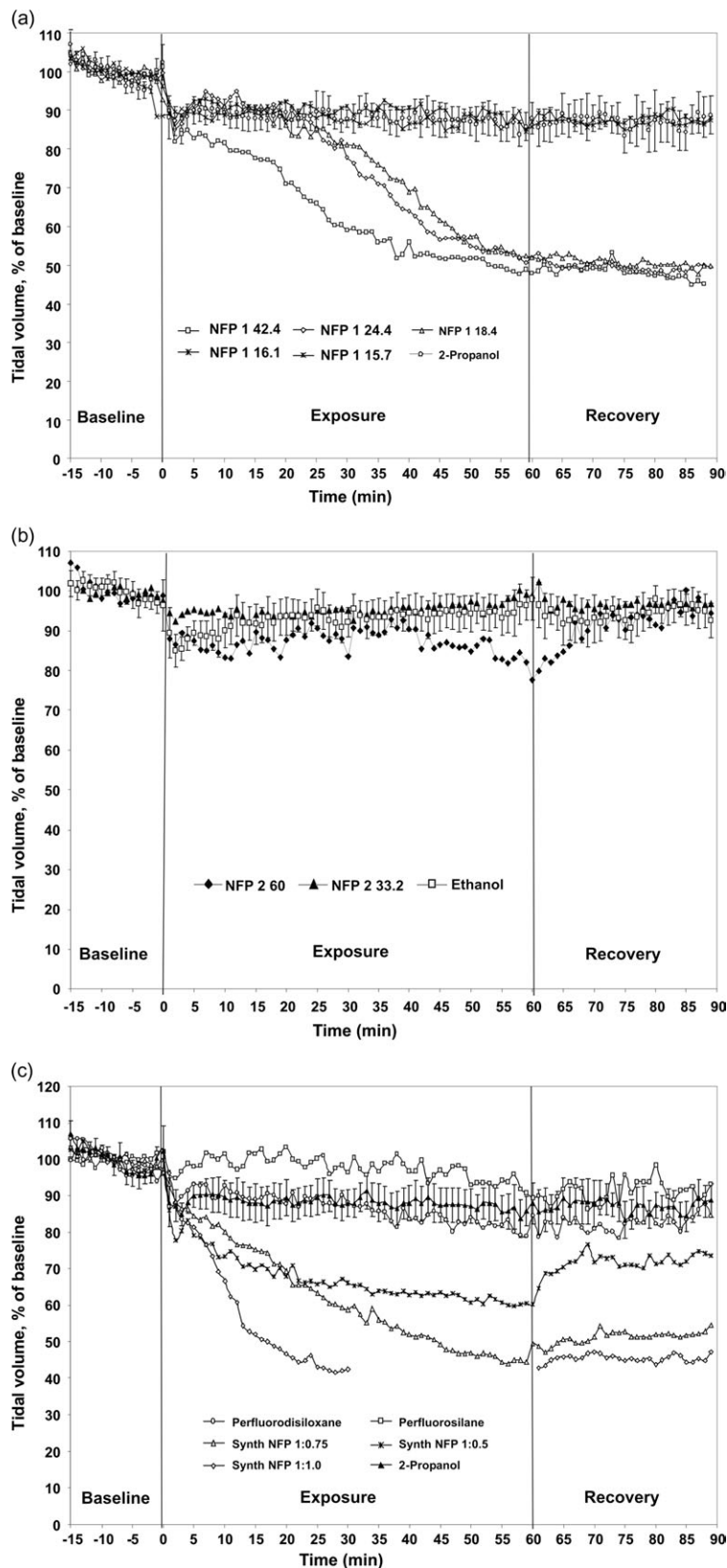
**FIG. 3.** Mice were exposed 15 min to laboratory air followed by a 60-min exposure to NFP 1 (a), NFP 2 (b), or NFP-related substances (c). In the recovery period, mice were allowed to breathe laboratory air for 30 min after the exposure. Respiratory parameters are VT. The vehicle control groups are depicted with their 95% confidence intervals. For clarity, only a limited number of representative time-response curves are shown. Exposure concentrations (milligrams/cubic meter) are given for NFP 1 and 2 (a and b, respectively).
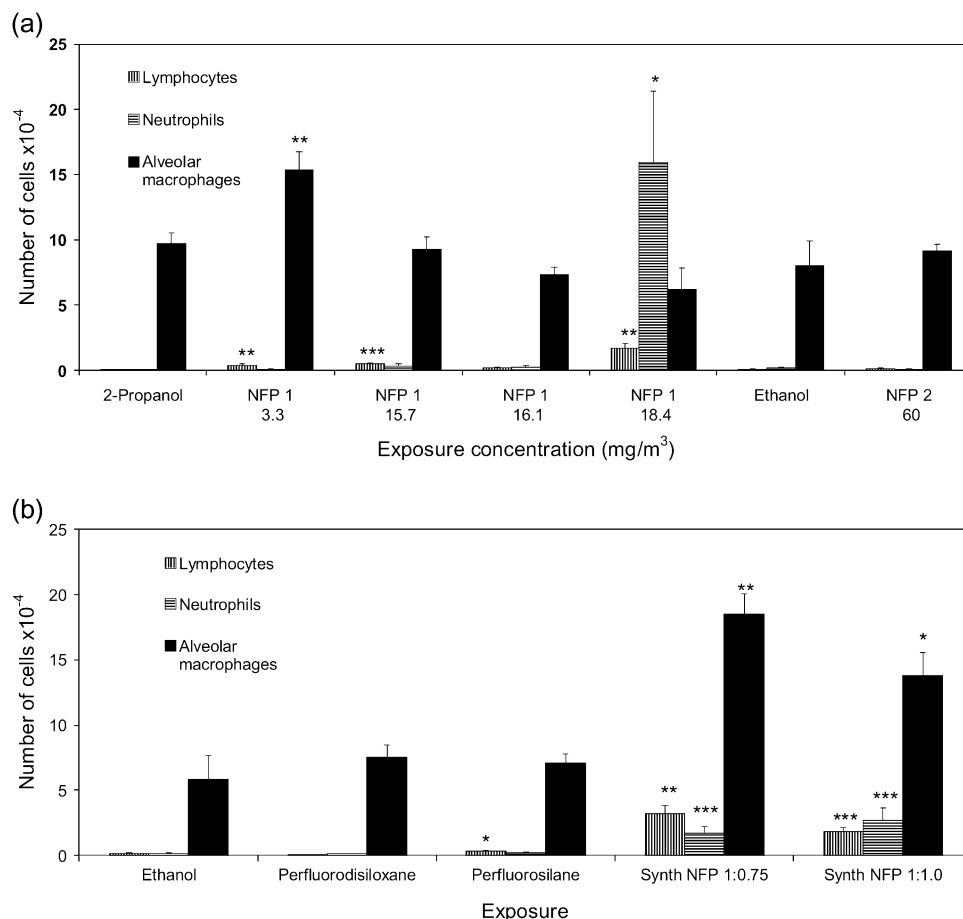
**FIG. 4.** Cells in BALF. Mice were lavaged by 4 × 0.8 ml saline 24 h after exposure to NFPs (a) or NFP-related substances (b). Bars are mean values with SEM. Group sizes are given in Table 2. Statistical significant differences from vehicle control groups are indicated by *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$.

*Statistics.* The effects of the NFPs on the respiratory parameters were compared with effects from the solvent controls. Statistical significance was accepted if the response induced by NFP exposure was not included in the 95% confidence interval of the vehicle control animals (cf. Fig. 3). In order to minimize the number of statistical tests, Kruskal-Wallis tests were applied to evaluate exposure-dependent effects on body weights, BAL cell counts, and protein in BALFs, respectively. If statistically significant differences were found ($p < 0.05$), data were further analyzed by the Mann-Whitney's *U*-test. Data in the NFP groups were compared pairwise with the corresponding responses in the vehicle control animals by means of the Mann-Whitney's *U*-test. A significant difference was accepted at $p$ levels below 0.05. Calculations were performed using the Minitab Statistical Software, Release 14 Xtra (Minitab Inc., PA).

## RESULTS

### Exposure Characterization

Table 1 summarizes inlet flow rates and the corresponding particle number and mass concentrations in the exposure chamber. The CPC, FMPS, and APS particle number and mass concentrations all increased linearly ($R^2 > 0.96$) with increased inlet concentrations. Therefore, particle numbers and particle size distributions were estimated by extrapolation by means of

linear regression analysis above the saturation level of the instruments (inlet flows above 0.1 ml/min). By number, the exposure concentrations were comparable for the two tested NFPs and chemicals ranging from $1.6 \times 10^5$ to $1.4 \times 10^7$ particles/cm$^3$ (FMPS + APS) at inlet flows of 0.01 to 0.5 ml/min liquid product. The FMPS measured slightly higher concentrations than the CPC ($\sim 1.4 \times 10^5$ to $4.6 \times 10^6$ particles/cm$^3$).

Gravimetric measurements showed a range in exposure concentrations from 3.3 to 42.4 mg/m$^3$ for NFP 1 and from 1.1 to 60 mg/m$^3$ for NFP 2. For NFP 1, the theoretical mass concentrations calculated from FMPS and APS data correlated with the gravimetric measurements (filter mass = 0.97 × calculated mass; $r^2 = 0.99$). The correlation in the case of NFP 2 was as follows: filter mass = 1.4 × calculated mass; $r^2 = 0.96$ (Table 1). Filter sampling of synth NFP 1:0.75 resulted in a particle concentration of 46.3 ± 0.4 mg/m$^3$ (inlet flow: 0.5 ml/min). This agrees with 42.4 ± 1.1 mg/m$^3$ measured for NFP 1 at the same inlet flow. Perfluorosilane and perfluorodisiloxane could not be sampled quantitatively by collection on filters because of their volatility. Thus, these data are absent in Table 1.

The particle size distribution of the two NFPs in addition to those of synthesized NFP 1, bis(1H,1H,2H,2H-perfluorooctyl) tetramethyldisiloxane, and the two NFP solvents (2-propanol and ethanol) are shown in Figure 2. All particle spectra are shown for an inlet flow rate of 0.05 ml/min.

It is clear that the NFPs generate characteristic size distributions with strong contributions of particles larger than 20 nm. NFP 1 produces smaller particles (peak size = 60.4 nm) than NFP 2 (peak size = 124 nm). Both NFPs also appear to produce a small mode ~10 and ~34 nm. The contribution of particles from 2-propanol and ethanol is minor, which is dominated by sizes below 200 nm with a peak in particle concentration at ~10 and ~34 nm. Consequently, the 10- to 34-nm particles found in the two commercial NFPs may be ascribed to residuals from the solvents. By number, the particle contributions reached from spraying pure solvents were $2.9 \times 10^4$ to $4.7 \times 10^4$ particles/$cm^3$ as compared with $1.4 \times 10^6$ to $1.6 \times 10^6$ particles/$cm^3$ spraying NFP 1 and NFP 2, respectively (all at inlet flows of 0.05 ml/min).

### Acute Airway Effects

*Exposure to the floor sealant, NFP 1*. A significant concentration-dependent decrease of the VT was observed during exposure (Fig. 3). Onset of the VT response occurred faster at higher concentrations and developed gradually. Above 16.1 mg/$m^3$, the VT reached the same plateau after 60 min of exposure. This indicates an abrupt response with a very steep exposure-response relationship. The response did not resolve during the 30-min recovery period. No clear exposure-dependent effect was seen on the respiratory rate (data not shown). Furthermore, the time of brake and the time of pause were unaffected, i.e., neither sensory nor pulmonary irritation was apparent from the respiratory tract (data not shown).

One day after the exposure, VT was still significantly suppressed in the 18.4 mg/$m^3$ group. Several mice in the 24.4 and 42.4 mg/$m^3$ groups were in a moribund state and consequently euthanized few hours after the exposure, why respiratory data are unavailable from these mice 1 day after NFP exposure.

*Exposure to tile coating, NFP 2*. Only the highest exposure concentration, 60 mg/$m^3$, gave rise to VT effects that were statistically significantly different from the vehicle control group. The effect was, however, relatively weak and reversible (cf. Fig. 3b).

*Exposure to perfluorosilane and perfluorodisiloxane*. Exposure to these compounds gave rise to little or no effect on the pulmonary function. Reductions in VT did, only to a limited extent, differ from the 95% confidence interval for the solvent control group, see Figure 3c. Furthermore, no effect was seen the day after the exposure.

*Exposure to synthesized perfluorosilane*. A gradual decrease in VT was seen after exposure to synthesized perfluorosilane with 0.5, 0.75, and 1.0 mol of water added for each hydrolyzable group (designated synth NFP 1: 0.5,

### TABLE 2
### Effects of Exposure on Body Weight and Protein in BALF

| Exposure | Number of mice in group | $\Delta m$ 22–24 h[a] (wt%) | Protein in BALF (µg/ml) |
|---|---|---|---|
| NFP 1 solvent control (2-propanol) (mg/$m^3$) | | | |
| 1 | 10 | 0.2 ± 1.0 | 163 ± 54 |
| NFP 1 (mg/$m^3$) | | | |
| 3.3 | 9 | 0.5 ± 1.2 | ND |
| 15.7 | 19 | 1.3 ± 1.2* | 143 ± 119 |
| 16.1 | 10 | 2.3 ± 1.4** | 133 ± 76 |
| 18.4 | 7 | 9.1 ± 3.9*** | 1153 ± 856** |
| NFP 2 solvent control (ethanol) (mg/$m^3$) | | | |
| 1.5 | 10 | 2.0 ± 1.0 | 118 ± 71 |
| NFP 2 (mg/$m^3$) | | | |
| 33.2 | 10 | 0.5 ± 1.5* | 207 ± 136 |
| 60.0 | 20 | 1.3 ± 0.9 | 144 ± 70 |
| Perfluorodisiloxane (mg/$m^3$) | | | |
| 26.5 | 10 | 1.3 ± 1.2 | 165 ± 48 |
| Perfluorosilane (mg/$m^3$) | | | |
| 1.8 | 8 | 2.0 ± 2.0 | 156 ± 44 |
| Synth NFP 1[b] | | | |
| NFP 1:0.5[c] | 8 | −0.1 ± 1.4 | — |
| NFP 1:0.75 46.3 mg/$m^3$ | 10 | 9.5 ± 2.2*** | 893 ± 311*** |
| NFP 1:1.0[c] | 10 | 8.5 ± 1.1*** | 743 ± 187*** |

*Note*. ND, not done.
Statistical significant differences from solvent control groups are indicated by *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$.
[a]Loss in body weight (% with SD) 22–24 h post exposure.
[b]Inlet flows were 0.5 ml/min in all tests with Synth NFP 1.
[c]No filter measurement.

0.75, and 1.0, respectively). Exposure to synth NFP 1:0.5 gave rise to an ~40% reduction in VT, whereas synth NFP 1:0.75 reduced the VT by ~55% (cf. Fig. 3). For both synth NFP 1:0.5 and synth NFP 1:0.75, the effect on VT was, at least in part, reversible (Fig. 3c). Exposure to synth NFP 1:1.0 gave rise to significant depression in the VT almost immediately after the onset of exposure. The exposure was abrogated after 30 min. At this time point, the mice were severely affected, and the exposure was discontinued for ethical reasons.

*Inflammatory cells and proteins in BALF*. Exposure to NFP 1 induced neutrophilic as well as lymphocytic lung inflammation that reached significantly increased numbers at an exposure to 18.4 mg/$m^3$ ($2.3 \times 10^6$ particles/$cm^3$) (Fig. 4a). Although the number of macrophages was increased in the lowest NFP 1 exposure group (3.3 mg/$m^3$), the number of macrophages did not differ from the control group at higher exposure concentrations. Mice exposed to 24.4 or 42.4 mg/$m^3$ were euthanized few hours post exposure to avoid unnecessary suffering; thus, no BAL data were obtained. No effects were seen on BAL cell numbers after exposure to NFP 2.

Mice exposed to perfluorosilane had increased number of lymphocytes (Fig. 4b). Exposure to synthesized perfluorosilane induced increased number of neutrophils, lymphocytes, and
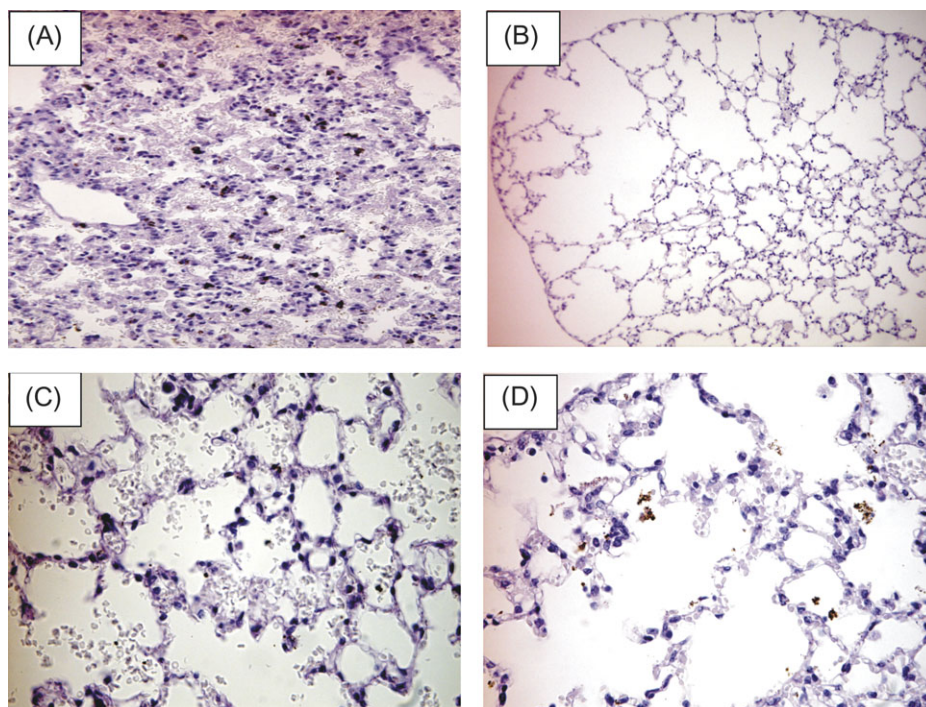
**FIG. 5.** Histological examination of lungs fixed with formalin 22–24 h after exposure to NFP 1 (42.4 mg/m$^3$). The observations were atelectasis (A), emphysema (B), and hemorrhage in the alveoli and bronchioles (C), and deposits of NFP in the alveoli, either free in the lumen or taken up by macrophages (D).

macrophages (Fig. 4b). No effects were seen after exposure to perfluorodisiloxane. Regarding total protein, increased levels were seen in BALFs from mice exposed to 18.4 mg/m$^3$ NFP 1, synth NFP 1:0.75, and synth NFP 1:1.0 (Table 2).

*Body weight.* Exposure to NFP 1 gave rise to concentration-dependent decreases in body weight. Body weights were significantly reduced in mice exposed to 15.7 mg/m$^3$ or more NFP 1 (Table 2). Furthermore, significantly reduced body weights were seen in mice exposed to synth NFP 1:0.75 and synth NFP 1:1.0 (cf. Table 2). No changes in body weights were seen in mice exposed to NFP 2.

*Histological examination of lung tissue.* The most extensive morphological damage was observed in the group exposed to NFP 1. Typical findings were emphysema-like morphological changes probably secondary to overdistension caused by atelectasis (Figs. 5A and 5B). Moreover, there were hemorrhages both in the alveoli and in the bronchioles (Fig. 5C) and damage to the type 1 pneumocytes. Particles from inhaled NFP 1 could be observed as brown deposits in the alveoli, either free in the lumen or taken up by alveolar or interstitial macrophages (Fig. 5D). In the bronchioles, the Clara cells had an "empty" appearance, suggesting the release of stored mucin.

## DISCUSSION

A number of severe cases of lung injuries have been associated with the use of spray sealing products (e.g., Pauluhn et al., 2008; Vernez et al., 2006). However, the causative agents have not yet been identified. It has been suggested that the mechanism of action could be related to alteration of surface tension of the lung lining fluid by perfluorinated polymers (cf. Vernez et al., 2006). Thus, to investigate the effect of perfluorination, we have measured the lung effects from two different NFPs, one with perfluorinated silanes/alkylsiloxanes (NFP 1) and another with nonfluorinated alkylsilanes/alkylsiloxanes (NFP 2).

A single 60-min exposure to NFP 1 at concentrations of 18.4 mg/m$^3$ and above induced a prominent impairment of the lung function in mice; this effect was not reversible over a period of 1 day. The effect was concentration dependent with a narrow window between no–observed effect level and lethal concentration. Similar effects could be observed for synthesized NFP 1, and the degree of hydrolysis of the fluorosilane was found to be a critical factor.

Experiments with 0.5, 0.75, and 1.0 mol water added for each hydrolyzable group in the fluorosilane showed significant increases of the toxic effects mimicking the effects of NFP 1. Thus, the effect appears to depend on the degree of hydrolysis forming free hydroxyl groups in the final product. This is also supported by the absence of effect from the nonhydrolyzed perfluorosilanes 1H,1H,2H,2H-perfluorooctyl triethoxysilane and bis-(1H,1H,2H,2H-perfluorooctyl) tetramethyldisiloxane and NFP 2.

Our study suggests that a combination of perfluorination and the number of free hydroxyl groups determines the toxicity of

NFP 1. Furthermore, protein in BALF was highly increased after exposure to NFP 1, indicating a toxic effect on the lung tissue. Furthermore, animals exposed to NFP 1 lost up to 10% of their body weight within the first 24 h after exposure, which suggests that the general health of the animals was considerably affected. The toxicity of NFP 1 was further confirmed by histological examination of the lung tissue. Inhalation of NFP 1 gave rise to atelectasis (collapsed alveoli), hemorrhage, and emphysema or lung overdistension because of maldistribution of ventilation. The effects gave rise to severely reduced lung function.

In conclusion, inhalation of nanofilm floor-sealing product with perfluorosilane induced acute and severe lung damage in mice. The degree of hydrolysis/condensation process is critical. Before a risk assessment of aerosolized surface-coating products can be carried out, it is warranted to investigate the underlying chemical and toxicological mechanisms that are responsible for the lethal lung effect.

For consumer protection, the results of our study highlight the relevance of taking the application procedure into account when assessing health aspects related to the use of spray products.

## ACKNOWLEDGMENTS

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