Lung lining liquid modifies PM$_{2.5}$ in favor of particle aggregation: a protective mechanism

MICHAELA KENDALL, TERESA D. TETLEY, EDWARD WIGZELL, BERNIE HUTTON, MARK NIEUWENHUIJSEN, AND PAUL LUCKHAM

1Imperial College of Science, Technology, and Medicine, London SW7 2BP; 2National Heart and Lung Institute, Imperial College of Science, Technology, and Medicine, London SW3 6LY; and 3Christopher Ingold Laboratories, Department of Chemistry, University College London WC1H 0AJ, United Kingdom

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Kendall, Michaela, Teresa D. Tetley, Edward Wigzell, Bernie Hutton, Mark Nieuwenhuijsen, and Paul Luckham. Lung lining liquid modifies PM$_{2.5}$ in favor of particle aggregation: a protective mechanism. Am J Physiol Lung Cell Mol Physiol 282: L109–L114, 2002.—The health effects of particle inhalation including urban air pollution and tobacco smoke comprise a significant public health concern worldwide, although the mechanisms by which inhaled particles cause premature deaths remain undetermined. In this study, we assessed the physicochemical interactions of fine airborne particles (PM$_{2.5}$) and lung lining liquid using scanning electron microscopy, atomic force microscopy, and X-ray photon spectroscopy. We provide experimental evidence to show that lung lining liquid modifies the chemistry and attractive forces at the surface of PM$_{2.5}$, which leads to enhanced particle aggregation. We propose that this is an important protective mechanism that aids particle clearance in the lung.

fine particles; bronchoalveolar fluid; surface chemistry; interactions

There is strong epidemiological and toxicological evidence that demonstrates the adverse health effects of atmospheric particulate matter having an aerodynamic diameter of $\leq 2.5 \mu$m, which is termed PM$_{2.5}$. Although the biological mechanism is not yet clear, particle size is believed to be a crucial factor in affecting health (13, 18). Particle size determines the site of deposition, the surface area-to-volume mass ratios, and importantly, the clearance rates within the respiratory tract. Thus smaller particles are more likely to reach the gas-exchange region of the lung and present a greater interactive surface per unit mass of inhaled material. In addition, although particles $>5 \mu$m in diameter undergo macrophage phagocytosis and mucociliary clearance, ultrafine particles ($<0.1 \mu$m in diameter) are not readily phagocytosed and may access the pulmonary interstitium via the epithelium (2, 9). Significantly, epidemiological studies of the effects of aerosols of different origins imply that bulk particle composition is a relatively poor predictor of health outcome compared with mass concentration (4) even though particle-surface chemistry is likely to be extremely significant in determining ultimate biological reactivity.

The first line of defense against inhaled particles is lung lining liquid, which bathes the underlying epithelial cells and contains important neutralizing agents including antioxidants, lysozyme defensins, lipids, mucus, and proteins. This liquid helps to maintain homeostasis of the airways through antimicrobial and immunologic defense mechanisms. An essential property of pulmonary surfactant, which is the predominant component of lung lining liquid in the respiratory units, is the capacity to reduce surface tension in an area-dependent way thereby preventing alveolar collapse. In addition, surfactant has been shown to displace respirable particles ($<6 \mu$m in diameter) into the hypophase of lung lining liquid, which makes the particles available for clearance by the mucociliary escalator (6, 15). Lung lining liquid proteins such as surfactant proteins A and D appear to be important modulators of the clearance of microorganisms by macrophage phagocytosis (3, 7, 12, 14, 22). Similar processes, possibly involving opsonization, may also trigger uptake by macrophages and clearance of inhaled PM$_{2.5}$.

The factors that render individuals susceptible to increased ambient PM$_{2.5}$ are also unclear, although those with existing respiratory and cardiovascular disease are most at risk during pollution episodes (17). Certainly the large surface areas presented to the peripheral lung by PM$_{2.5}$ have enormous potential to deliver toxic material to or deplete defensive material from the lung lining fluid. If one role of the fluid is to prevent such toxic processes, abnormalities in lung lining liquid composition, e.g., in smokers and asthmatics (7, 8), may contribute to the observed susceptibility to PM$_{2.5}$ episodes. However, very little is understood about the interaction between lung lining liquid and inhaled ambient particles.

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In this study, we hypothesized that in healthy individuals the lung lining liquid plays an important protective role by modifying the physicochemical properties at the surface of particulate matter and thus changing the surface chemistry and neutralizing its reactivity in situ. We also hypothesized that these modifications act to enhance pulmonary clearance by increasing the attractive forces between the particles, thereby inducing aggregation and enhanced phagocytosis by macrophages. To investigate this, we used scanning electron microscopy (SEM), atomic force microscopy (AFM), and X-ray photon spectroscopy (XPS) to show that lung lining liquid modifies the surface chemistry and attractive forces at the surface of PM$_{2.5}$, which leads to enhanced particle aggregation. We used SEM to determine particle morphology before and after immersion in human lavage fluid, AFM to examine particle-liquid interactions, and XPS to examine changes in particle-surface chemistry before and after immersion in lavage fluid.

**MATERIALS AND METHODS**

Lung lining liquid was collected using bronchoalveolar lavage. Human bronchoalveolar lavage fluid (BALF) was collected during diagnostic fiber-optic bronchoscopy with a routine method as described previously (21). Briefly, the tip of the bronchoscope was wedged into a segment of the right middle lobe. Warmed sterile 0.15 M NaCl (50 ml) was introduced and gently aspirated. This was repeated three times (total lavage, 200 ml), and the washings were pooled and centrifuged at 300 g for 10 min to remove the cellular component, which was used for diagnostic purposes. The supernatant was stored at −40°C until used. Because a large volume of lavage fluid was required to perform the whole experiment, the supernatant from two subjects (who were subsequently diagnosed as normal) were pooled before the study.

PM$_{2.5}$ was collected from three sources, which represented outdoor urban air, outdoor “clean” air, and indoor tobacco smoke pollution. Atmospheric or outdoor PM$_{2.5}$ was collected near London traffic (”urban PM$_{2.5}$”) and at a clean atmosphere-monitoring station in Mace Head, Galway (“clean air PM$_{2.5}$”). “Indoor smoke PM$_{2.5}$” was collected in an indoor smoking area. Personal PM$_{2.5}$ samplers (BGI-400 pump fitted with GK-2.05 cyclone; BGI, Waltham, MA) were used to collect PM$_{2.5}$ onto Nucleopore and Teflon filters for subsequent SEM and XPS analysis, respectively (11). In addition, a purpose-designed, direct particle-liquid (DPL) system was configured to allow collection of PM$_{2.5}$ directly into liquid, in this case 0.15 M NaCl or BALF. This system is based on an existing bioaerosol sampler design and a more complete description can be found elsewhere (10). Table 1 summarizes the types of samples taken as part of this study, the collection methods used, and the associated analytic methods employed.

The filter samples collected with personal PM$_{2.5}$ samplers were stored in airtight containers in a cool dark room for up to 4 days. Using a scalpel, the filters were then divided into three parts. One unchanged (dry) portion was analyzed using XPS, another portion was immersed in sterile 0.15 M NaCl, and the third was immersed in BALF, for 4 h. The saline- and lavage-treated samples were then bathed in nanopure water and placed in a desiccating argon atmosphere for 24 h. Samples collected by DPL were aspirated from the collection reservoir, and the supernatant was filtered through a 0.4-μm-pore-size Nucleopore filter under negative pressure. Samples were centrifuged immediately after collection at 3,000 rpm for 30 min to remove the largest particles. The filters were placed in a desiccating argon atmosphere for 24 h to dry before SEM analysis.

AFM was performed on diesel exhaust particles of ~10 μm in diameter, because particles collected as PM$_{2.5}$ were too small to manipulate accurately. Exhaust particles were collected onto a glass slide securely fixed inside a clean container. The container was opened and held at the exhaust outlet of a diesel engine to allow particles to strike the glass slide. The container was then sealed and taken to the laboratory where the slide was examined under a microscope to identify single compact 10-μm particles. Force-distance measurements were carried out with a Topoetrix Explorer 2000 (ThermoMicroscopes) using an Ultralever contact tipless cantilever (Park Scientific Instruments). A cantilever with the tip primed with a small amount of epoxy resin adhesive was maneuvered using a micromanipulator over a previously identified particle and lowered onto it; the cantilever was then moved away from the slide and viewed under a microscope to confirm that the particle was satisfactorily attached to the cantilever. In addition, the particle was examined to ensure that it was not too “flat” and that the contact area was not covered in resin. The preparation was allowed to dry for 48 h before study. A carbon graphite disc (20 mm in diameter) was used as the contact surface.

XPS was used to determine the surface chemistry of PM$_{2.5}$ to a depth of ~5 nm. XPS measurements were performed on a VG ESCALAB 220i XL instrument using monochromatic Al-Kα radiation (1,486.6 eV) of 600-μm spot size. A magnetic objective lens was used for enhanced sensitivity throughout this work, and the analysis chamber was maintained at a pressure <10$^{-9}$ Torr. Survey spectra of the particulates were collected over a 1,100-eV range at a resolution of 0.8 eV/step and 100 ms/step and a pass energy of 100 eV. High-resolution spectra were collected for species of interest at a resolution of 0.1 eV/step and 100 ms/step and a pass energy of 20 eV. Charge compensation was achieved by placing a tantalum-conducting mask over the sample to ensure good electrical contact before flooding the sample with low-energy (4 eV) electrons. All peaks were referenced to the C1s binding energy for hydrocarbons at 285.0 eV. Quantification was performed using a Shirley background (19) and the sensitivity factors described previously (23). Binding energies were taken at peak maxima for all species. Particles were collected on Teflon filters and analyzed before and after immersion in

<table>
<thead>
<tr>
<th>Collection Method/Analytical Method</th>
<th>Teflon Filter/Chemical (XPS)</th>
<th>Nucleopore Filter/Morphological (SEM)</th>
<th>DPL System/Morphological (SEM)</th>
<th>Glass Slide/Physicochemical (AFM)</th>
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<tr>
<td>Clean air PM$_{2.5}$</td>
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Table 1. Summary of types of particle samples collected and analytical methods used

Fig. 1. Typical particles observed by scanning electron microscopy (SEM) of particulate matter with <2.5-μm diameter (PM_{2.5}) samples collected directly onto Nucleopore filters from the London atmosphere (A–C) and an indoor smoking area (D–E). These two particle types are similar in appearance and comprise agglomerated ~35-nm spherules generated during combustion. Particles may even appear fibrous because of single-particle chain agglomeration (C). Smallest particle type found in the clean air sample is shown (F); compared with combustion particles, the particles sampled at this site tended to be larger in size, crystalline in structure, and tended to occur at much lower concentrations. Dark holes on all images are the filter pores.
normal saline and unprocessed lavage fluid. Displacement of some of the atmospheric particles from the Teflon filters was detected after immersion in saline and lavage fluid. Blank Teflon filters were therefore used to establish the background Teflon spectra and to insure that there was no appreciable surface contamination during liquid treatments.

RESULTS

Representative SEM images of the atmospheric PM$_{2.5}$ samples collected onto Nucleopore filters are shown in Fig. 1. Figure 1, A–C, shows typical examples of urban particles that are characteristic of those found in the U.K. urban atmosphere as previously reported (1, 20). Figure 1, D and E, shows typical indoor smoke particles. Both sample types are dominated by small particles comprised of loosely agglomerated 35-nm spheres that form chains or transparent particles. Figure 1F shows typical particles in the clean air sample; compared with the urban particle samples, these particles tend to appear as larger single crustal particles that are denser in structure and occur at much lower concentrations.

When the urban particle samples were collected using the DPL sampler in the presence of BALF, the most striking feature was the appearance of increased numbers of dense conglomerates >5 $\mu$m (mostly in the 10-$\mu$m range) that consisted predominantly of agglomerated 35-nm particles (Fig. 2, A and B). This particle size (~10 $\mu$m) was largely absent in ambient air samples and in the saline DPL control sample, where most of the particles were 35-nm spheres and small-chain agglomerates (~2.5 $\mu$m) with the occasional less-dense larger particles that may have formed during sample drying. In 10 randomly selected fields of view, more than twice as many large- and medium-sized agglomerates appeared in the BALF-collected sample. Figure 3 shows two low-magnification backscatter SEM images of the BALF- and the saline-collected particles and shows the relative abundance of large, densely packed agglomerates, which appear as dark patches. Clearly, the BALF-collected particles agglomerated, whereas particles collected into the saline sample remained dispersed.
AFM-measured force-distance values for the interaction forces between the particle and the surface in different media are presented in Fig. 4 under atmospheric conditions (A), in nanopure water (B), and in processed BALF (C). When a particle was brought toward the graphite surface in air, there was a short-range attraction due to van der Waals forces. The adhesive force observed on separation in air likely reflects van der Waals forces and/or capillary bridging.

In water, there was a marked reduction in these forces, possibly due to a slight repulsion between the surfaces and reduced van der Waals interaction as would be expected in liquid. When BALF was examined without processing (i.e., centrifugation), the opaque, lipid-rich fluid interfered with the force measurements by attenuating the path of the laser in the AFM. Consequently, the lavage sample was centrifuged to remove most of the lipid component, which left a protein-rich supernatant. Treatment of particles with processed BALF resulted in longer range attraction and adhesion forces than was observed in air or water.

XPS analysis confirmed that very low particle loads were present in the clean air sample. Particles from this site consisted of graphite-hydrocarbon, Cl/H, and oxide species only. Higher particle loads were observed for the outdoor urban and indoor smoke samples. Graphite-hydrocarbon species dominated at the particle surface, although lower levels of SiO, oxide, and amide species were also present. In addition, trace species on urban outdoor-particle surfaces included O/N and C=O/COO, Cl-, NO, NH, and SO4-. After immersion in saline, Cl-, NO, NH, and SO4 were no longer observed at the surface and were deemed bioavailable. After immersion in BALF, a strong amide signal was observed on the particles, which indicates that significant quantities of protein had adsorbed onto the surface of these particles. No amide signal was recorded on the blank filter control or the saline control. Figure 5 shows the detected amide signal from urban PM2.5: dry PM2.5 (A), saline-soaked (B), and bronchoalveolar lining fluid-soaked (C) urban PM2.5, using X-ray photon spectroscopy.

DISCUSSION

In this study, we have shown that when PM2.5 is collected directly into normal lung lining liquid, the particles aggregate into larger (>5 μm) dense structures compared with samples collected in air or into saline. The control showed that the agglomeration effects were not due to drying per se but were specifically associated with the protein-rich solution, which is in
line with the AFM study that showed enhanced attraction between surfaces in BALF. The XPS studies of surface chemistry for urban and smoking PM2.5 showed significant modification by BALF, together with the AFM findings of increased attractive and adhesive forces in BALF suggest that aggregation is enhanced by components of lung lining liquid. The transition of PM2.5 surface chemistry from a principally organic carbon layer with trace soluble species to an organic layer with no trace soluble species and a strong amide signal indicates that the surface adsorption of protein from lung lining liquid may be responsible for this change. The long-range molecular attractions shown to occur between a particle and a graphite surface in protein-rich BALF may be attributable to particle surface-protein interactions. Opsonization of inhaled particles by surfactant proteins [which are known to have opsonizing properties (12, 22)], antioxidants, and serum-derived and other locally produced proteins may change the surface-charge forces in favor of aggregation. The processed BALF used in this study may represent the hypophase of lung lining liquid (described in Refs. 7 and 16) into which the surface surfactant film has been shown to submerge particles <6 μm (15, 16). It is suggested that when the particles reach the hypophase, they are then more readily cleared by normal clearance mechanisms.

This aggregation mechanism is highly significant because macrophages do not readily phagocytize the smaller agglomerates of 35-nm spheres that dominate urban air in developed countries (1); epithelial cells have been demonstrated to internalize these ultrafine particles (9). We hypothesize that in susceptible subjects, the inability of PM2.5 to aggregate in lung lining liquid, which is possibly due to low opsonization, reduces the chances of particle clearance by macrophages and enhances the possibility of epithelial cell uptake and transfer to the interstitium (2, 9). In addition, particle adsorption and depletion of lung lining components, for example surfactant components such as proteins and antioxidants, may compromise lung defense mechanisms. Such processes may contribute to susceptibility to PM2.5 in elderly patients with existing cardiovascular and respiratory diseases and may result in the acute increased mortality rates observed during PM2.5 pollution episodes.

We thank John Watt (Middlesex University) for producing Fig. 3, A and B.
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REFERENCES