Adsorption of surfactant protein D from human respiratory secretions by carbon nanotubes and polystyrene nanoparticles depends on nanomaterial surface modification and size

Magda Marchetti1,2, Milo S. P. Shaffer3, Martina Zambianchi1, Shu Chen4, Fabiana Superti2, Stephan Schwander5, Andrew Gow6, Junfeng (Jim) Zhang7, Kian Fan Chung1, Mary P. Ryan4, Alexandra E. Porter4 and Teresa D. Tetley1

1National Heart and Lung Institute, Imperial College London, Dovehouse St., London SW3 6LY, UK
2Department of Technology and Health, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
3Department of Chemistry and London Centre for Nanotechnology, and 4Department of Materials and London Centre for Nanotechnology, Imperial College London, Exhibition Road, London SW7 2AZ, UK
5Department of Environmental and Occupational Health, Rutgers School of Public Health, Piscataway, NJ 08854, USA
6Department of Pharmacology and Toxicology, Rutgers University, Piscataway, NJ 08854, USA
7Nicholas School of the Environment, Duke University, Durham, NC 27708, USA

The alveolar respiratory unit constitutes one of the main targets of inhaled nanoparticles; the effect of engineered nanomaterials (NMs) on human health is largely unknown. Surfactant protein D (SP-D) is synthesized by alveolar type II epithelial cells and released into respiratory secretions; its main function is in immune defence, notably against inhaled microbes. SP-D also plays an important role in modulating an appropriate inflammatory response in the lung, and reduced SP-D is associated with a number of inflammatory lung diseases. Adsorption of SP-D to inhaled NMs may facilitate their removal via macrophage phagocytosis. This study addresses the hypothesis that the chemistry, size and surface modification of engineered NMs will impact on their interaction with, and adsorption of, SP-D. To this purpose, we have examined the interactions between SP-D in human lung lavage and two NMs, carbon nanotubes and polystyrene nanoparticles, with different surface functionalization. We have demonstrated that particle size, functionalization and concentration affect the adsorption of SP-D from human lung lavage. Functionalization with negatively charged groups enhanced the amount of SP-D binding. While SP-D binding would be expected to enhance macrophage phagocytosis, these results suggest that the degree of binding is markedly affected by the physicochemistry of the NM and that deposition of high levels of some nanoparticles within the alveolar unit might deplete SP-D levels and affect alveolar immune defence mechanisms.

1. Introduction

The main function of pulmonary surfactant is to prevent alveolar collapse during breathing by reducing the surface tension at the air–liquid interface, but its role in the innate immune defence system of the lungs is also very important. Surfactant consists mostly of phospholipids, which play a major role in reducing surface tension, and approximately 10% protein. Four specific proteins associate with phospholipids to form the pulmonary surfactant of vertebrates, surfactant protein (SP)-A, SP-B, SP-C and SP-D. The hydrophobic SP-B and SP-C and hydrophilic SP-A help lower surface tension, associating with the phospholipids; SP-A and SP-D, also hydrophilic, are primarily involved in lung defence, as members of the collectin family. SP-D is composed of 12 subunits, each with a molecular mass of 43 kDa. It is a complex molecule: each subunit contains a...
carbohydrate recognition domain, the neck domain, a collagenous domain and an N-terminal cysteine-rich domain. The terminal carbohydrate recognition domain is important in agglutination and opsonization of a variety of viruses, bacteria and fungi. Moreover, it has been demonstrated that SP-D contributes to the biophysical properties of alveolar surfactant, maintenance of surfactant homeostasis and ultrastructure [1,2].

With the advent of nanotechnology, nanomaterials (NMs; which have at least one dimension measuring 100 nm or less) are now present in many commercial products and are used in medicine, particularly in innovative diagnostic and drug delivery systems. The putative effect of these engineered NMs on human health, either through environmental or intentional exposure, is still unknown, but it is believed that the pulmonary respiratory alveolar compartment constitutes one of the main targets of inhaled nanoparticles and is also an attractive site for nanodrug delivery [3].

Although several studies have investigated the adsorption of surfactant components on different NMs such as metal or metal-oxide nanoparticles (NPs) [4–7], polystyrene NPs [8] and carbon nanotubes (CNTs) [9–11], it is difficult to draw general conclusions on the subject as different surfactant models have been used in each study. Some have used semisynthetic or natural surfactant preparations that do not contain SP-A and SP-D, focusing mainly on the interaction with the lipidic components [5,4,10]. Others have used recombinant proteins [12], in the absence of other constituents of surfactant, which have important therapeutic applications but are generally truncated fragments that might lose some of the original biological function and exhibit different behaviour compared to the natural surfactant proteins [13]. Other studies have used SP-D isolated from bronchoalveolar lavage fluid (BALF) from patients with alveolar proteinosis, taking advantage of the high levels of surfactant and surfactant proteins in this condition [8,9,14], but there are significant differences between the profile of isoforms of SP-A isolated from these samples compared to those isolated from normal lung [15] and it is possible that the same is true for SP-D [16], which may influence NM–SP-D interactions.

Nevertheless, these studies of separate components of surfactant indicate that the interaction of NMs with pulmonary surfactant is an important consideration of their impact at the alveolar gas–liquid interface. In situ, surfactant will be the first biological structure with which NMs will interact in the alveolar compartment. This study addresses how the adsorption of SP-D from human lung secretions by NMs is affected by its size/shape and surface modification. We have examined the interaction of SP-D (present in human lung secretions from lung tissue with normal appearance) with both multiwalled carbon nanotubes (MWN Ts) and polystyrene latex spheres, each with three different surface functionalizations. Adsorption of SP-D to these materials was found to depend on the size, concentration and surface charge of the NMs.

2. Material and methods
(a) Multi-walled carbon nanotubes and polystyrene nanoparticles

The MWN Ts (diameter 12.1 nm ± 3.7 nm, length 700 nm), synthesized by chemical vapour deposition, were obtained from Arkema SA (Lacq-Mourenx, France) and have been thoroughly characterized [17]. The pristine, as-received MWN Ts were functionalized with poly(4-vinyl pyridine) (P(4VP)-MWNT) and acid oxidized (AO-MWNT) to provide three surface modifications [17]. Pristine MWNT are weakly acidic with an isoelectric point of 4.9; the P(4VP)-MWNTs have a nitrogen-containing group, shifting the isoelectric point to 7.0; acid oxidized AO-MWNTs present a generally acidic surface, with a complex mixture of functional groups present [17]. Prior to incubation with human lung secretions, MWN Ts were suspended in PBS at a concentration of 1 mg ml⁻¹ then sonicated in a sonicating water bath for 5 min and diluted to a concentration twice the desired final concentration.

Unmodified Fluoresbrite YG latex polystyrene particles 2.5% (100 nm), carboxyl-modified Fluoresbrite YG latex polystyrene particles 2.65% (100 nm) and carboxyl-modified Fluoresbrite YG latex polystyrene particles 2.6% (50 nm) were purchased from Polysciences. Amine-modified fluorescent orange latex polystyrene particles 2.5% (100 nm) and amine-modified fluorescent blue latex polystyrene particles 2.5% (50 nm) were purchased from Sigma Aldrich. Unmodified Dragon Green latex polystyrene particles 1% (50 nm) were purchased from Bangs Laboratories. The fluorescent markers are incorporated within the particles so that there is no effect of these markers on surface bioactivity. The carboxyl-modified NPs have an isoelectric point of pH 4–6 and amine-modified NPs have an isoelectric point at pH 8–10 (as published by the manufacturers). These NPs were sonicated in a sonicating water bath for 30 s and diluted in PBS at twice the desired final concentration.

(b) Human lung tissue and collection of lung lining liquid

Human lung lining liquid was collected from pieces of lung tissue obtained from patients undergoing lobectomy for carcinoma of the lung. Regions of lung well away from the tumour, with normal appearance, were perfused four times with 50 ml of Hank’s balanced salt solution for each 5–10 g tissue fragment (Invitrogen, Paisley, UK). The washes were then centrifuged at 300 g for 10 min to pellet and remove the cells, mostly macrophages. The supernatants were analysed for total protein using the Bradford assay. Protein samples were diluted 1:100 with Bradford assay solution to give a working volume of 200 μl. The optical density was measured at 570 nm and protein concentration was calculated from regression data generated from a BSA standard curve. The fractions with the highest protein content were chosen and SP-D concentration was determined semi-quantitatively using immunoblotting (described in §2e). The highest amount of SP-D was contained in the fractions with the highest protein content, which were either the first or second wash. These samples were used in the NM interaction studies.

(c) Preparation of surfactant protein D-rich human lung wash and interaction with nanomaterials

The selected human lung tissue wash (1 ml) was centrifuged at 10 000 g for 30 min at 4°C to separate the pellet (the surfactant and surfactant bound proteins, including lipid-bound SP-A: SP-A-rich fraction) from the soluble protein, which contains the water soluble SP-D, termed SP-D-rich surfactant [18]. SDS-PAGE and immunoblotting (described in §2d,e) of the two fractions confirmed that SP-D was in the supernatant only, while SP-A was in the pellet only (figure 1). The high SP-D-rich surfactant was aliquoted, stored at −80°C and used in all the subsequent experiments. To determine SP-D adsorption to NMs, 100 μl of the SP-D-rich surfactant was incubated with the same volume of increasing concentrations of MWN Ts.
Figure 1. SDS-PAGE and immunoblotting for SP-A and SP-D performed in the supernatant and the pellet of the lung wash. Human lung wash was centrifuged at 10 000g for 30 min at 4°C to separate the supernatant (S) from the pellet (P).

and polystyrene spheres for 1 h at room temperature, on a rotator. Final NM concentrations were 0, 0.1, 0.5, 1, 5, 10, 20 and 40 μg ml⁻¹. Samples were then centrifuged at 17 000g for 30 min to separate NMs and bound proteins from the unbound proteins. The supernatant was aspirated, while the pellet was resuspended and washed in PBS then centrifuged at 17 000g for 30 min to remove any unbound protein. The NM pellet and supernatant were processed as described in §2d,e. At least three experiments were performed for each sample.

(d) SDS-PAGE
NUPAGE LDS sample buffer (Invitrogen) and reducing agent were added to 13 μl of supernatant (corresponding to 15 μg total protein content). The NM pellets were resuspended in 13 μl of PBS. Samples were heated at 70°C for 10 min and loaded onto a 4–12% Novex Bis–Tris SDS-PAGE gradient gel (Invitrogen). Novex Sharp Pre-stained Protein Standard was used as the molecular mass marker (Invitrogen). The gel was run with MOPS running buffer at 200 V for 50 min.

(e) Immunoblotting
Following electrophoresis, the proteins were transferred to a nitrocellulose membrane using the iBlot system (Invitrogen). Membranes were washed with Tris-buffered saline containing 1% Tween20 (TBST), blocked for 1 h in TBST containing 5% skimmed milk and probed overnight for SP-A or SP-D using a polyclonal rabbit antibody (SP-A: 1 μg ml⁻¹, Abcam) or a mouse monoclonal antibody (SP-D: 0.05 μg ml⁻¹, Abcam). Membranes were washed three times in TBST for 5 min prior to incubation with an anti-rabbit (SP-A) or anti-mouse (SP-D) HRP-conjugated secondary antibody (Dako, Ely, UK). Membranes were then washed three times for 10 min prior to incubation with enhanced chemiluminescence (ECL) + solution (GE Lifesciences), and exposed to autoradiographic film. Band densities were quantified by GelDoc-It Imaging System and VisionWorksLS Image Acquisition and Analysis Software (UVP). The enhancement or reduction of the SP-D signal was compared to the NM-untreated controls. To determine whether nanoparticle migration influenced protein migration, the fluorescent polystyrene nanoparticles were visualized using appropriate light wavelengths and were observed to remain at the loading site; furthermore, protein migration matched that of non-particle-treated, control proteins.

(f) Statistics
Data are presented as mean ± s.e.m. Significance was tested using a one-way ANOVA with a Dunnet post test, to compare each sample to the control (lung washing without MWNTs/NPs). Significance was set at 0.05.

3. Results
The total protein content of the chosen lung wash was 2.32 μg ml⁻¹, about 100 times more than reported in BALF from patients with alveolar proteinosis [9], likely reflecting dilution of the alveolar proteinosis BALF due to pooling serial lavage fractions. The protein concentration of the lung wash used in this study did not change when the assay was performed on the supernatant after centrifugation to remove surfactant, indicating that most of the proteins were soluble, not associated with surfactant. Previous unpublished studies by us show that most of the surfactant is collected in the first three washes, so the technique used in this study should result in washes that are highly enriched with lung surfactant/secretions, and indeed, the SP-A and SP-D immunoblotting indicated this (figure 1). The high protein concentration of this early wash was not diluted by pooling sequential washes as occurs in conventional BAL procedures, although it is still a diluted sample of lung lining liquid, as washing is the only way to sample this fluid.

SDS-PAGE and immunoblotting for SP-A and SP-D were performed on the surfactant pellet and the supernatant of the lung wash. As expected, the pellet contained lipid-bound SP-A and the supernatant contained soluble SP-D, but SP-D was not detected in the pellet and SP-A was not detected in the supernatant using this technique (figure 1).

For pristine MWNTs, P(4VP)-MWNTs and AO-MWNTs (figure 2) there was a non-significant trend for SP-D binding to increase with increasing concentration of pristine and P(4-PV)-MWNTs, whereas with AO-MWNTs there was a significantly high degree of SP-D binding, at 5 μg ml⁻¹ and above, which increased with concentration of AO-MWNTs, to between 4.5- and 6-fold that detected in the non-particle control. Accordingly, when the SP-D level remaining in the supernatant fraction was examined, AO-MWNTs were the only nanotubes with which we observed a slight, but insignificant, decrease of the SP-D content at increasing MWNT concentrations compared with the untreated controls (data not shown).

When unmodified 100 nm polystyrene latex NPs were incubated with SP-D-enriched supernatants (figure 3), the NP-associated SP-D in the nanoparticle pellet [unmodified NPs (P)] tended to increase, though not significantly, as the NP concentration increased; however, there was no difference in the SP-D remaining in the supernatant [unmodified NPs (S)]. By contrast, for both amine-modified and carboxyl-modified polystyrene, the particle-bound SP-D increased with increasing concentration of polystyrene, reaching eightfold and 13-fold, respectively, of the non-particle control at the highest concentration of polystyrene, the particle-bound SP-D increased with increasing concentration of pristine and P(4-PV)-MWNTs, whereas with AO-MWNTs there was a significantly high degree of SP-D binding, at 5 μg ml⁻¹ and above, which increased with concentration of AO-MWNTs, to between 4.5- and 6-fold that detected in the non-particle control. Accordingly, when the SP-D level remaining in the supernatant fraction was examined, AO-MWNTs were the only nanotubes with which we observed a slight, but insignificant, decrease of the SP-D content at increasing MWNT concentrations compared with the untreated controls (data not shown).

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When the same experiment was performed with 50 nm polystyrene NPs, the only significant increase in SP-D adsorption was with the carboxyl-modified particles, which increased with concentration but only reached 1.8-fold that of the
In this study, we have shown that SP-D is adsorbed from the soluble protein fraction of human lung secretions by MWNTs (data not shown). There was no change in SP-D level in the supernatant fractions following treatment with the 50 nm polystyrene NPs (figure 4). However, considering its role in host defense from respiratory pathogens, there might be a higher susceptibility to microbial infection, thus facilitating clearance by, for example, macrophage phagocytosis, as suggested by others [8,12,11,19].

4. Discussion

In this study, we have shown that SP-D is adsorbed from the soluble protein fraction of human lung secretions by MWNTs and latex polystyrene NPs and that the degree of binding depends on surface charge difference and intensity, as well as the size of the NPs and amount of NM exposure. This finding has both positive and negative implications. SP-D binding to NMs would be expected to cause their aggregation, thus facilitating clearance by, for example, macrophage phagocytosis, as suggested by others [8,12,11,19]. However, considering its role in host defense from respiratory pathogens, there might be a higher susceptibility to microbial infection.
infection as a consequence of SP-D sequestration by NMs, depending on the amount deposited. In addition to its role in host defence, SP-D is involved in the maintenance of surfactant ultrastructure, lipid homeostasis and metabolism. In fact, SP-D−/− mice show a progressive accumulation of surfactant in the lungs, due to impaired uptake by alveolar type II cells [1], indicating an important role in modulating surfactant recycling. Therefore, deposition of NMs in the alveolar region could hamper the normal surfactant functions due to adsorption of SP-D. This study indicates that increasing the concentration of the NMs results in increased SP-D adsorption, suggesting that this could also occur, and be important, in vivo. Moreover, formation of a ‘surfactant protein corona’ [12,20] on NMs, might modify their destiny in the alveolar compartment, depending on the magnitude and profile of protein binding. This comparative study of the propensity of NMs to adsorb SP-D illustrates the importance of particle physicochemistry, and how, regardless of the material core, the size and charge of the NMs crucially affect this.

Manufactured single-walled carbon nanotubes (SWNTs) and MWN Ts elicit pathological changes in the lungs [21] of experimental animals. CNTs show very poor solubility and a tendency to agglomerate; however, aqueous processing would be advantageous in a wide range of CNT applications, especially in the biomedical field where they are being studied as drug delivery tools [22]. Despite known problems associated with MWNT damage and debris accumulation, oxidation using strong acids is the most common approach to maximize dispersibility, producing good aqueous dispersions with a high degree of functionalization with –COOH groups suitable for further modification [17]. This study shows that SP-D binds more strongly to the negative surface of AO-MWN Ts compared with the weakly negative pristine or the weakly positive P(4VP)-MWNTs. The isoelectric point of SP-D is 6–8, so at physiological pH the protein does not have a net charge but it presents positive and negative amino acidic residues on its surface. In particular, a large positively charged area has been described near the carbohydrate-binding site [23], which may account for its high affinity to AO-MWNTs. Since all the MWNTs were of the same length and diameter, the strong binding of SP-D to AO-MWNTs would not be expected to reflect very large differences in surface area, although our recent comparative work [17] indicates that the topography of the AO-MWNTs is very different from that of the pristine and P(4-PV)-MWNTs, being non-uniform, which may have further enhanced interaction with SP-D.

To better understand how positively or negatively charged groups on the surface of NMs affect SP-D binding, we extended the study to a well described NM model, polystyrene latex NPs, which we have fully characterized previously [24]. These NPs are widely used as a model to study interactions between NPs and biological structures for many reasons, including their commercial availability, high-quality, homogeneity and wide variety of size and surface chemistry [25]. We compared SP-D adsorption to unmodified, amine-modified and carboxyl-modified polystyrene NPs. We used two sizes (50 nm and 100 nm) to check the influence of the surface area on SP-D binding. At the same concentration (40 μg ml⁻¹, the highest used in this study), the number of 50 nm NPs (9.14 × 10⁶ NPs ml⁻¹), and consequently, the surface area (approx. 71 720 μm² ml⁻¹) are much greater compared to the 100 nm NPs (7.31 × 10⁸ NPs ml⁻¹; surface area 2295 μm² ml⁻¹) resulting in a total reactive surface that is approximately 31 times larger than the 100 nm NPs [24].

The results confirmed our observations with MWNTs, that functionalization with carboxylic groups results in the most enhanced SP-D binding. However, in contrast to the MWNTs, the positively charged 100 nm polystyrene NPs also adsorbed SP-D, though not as avidly as the carboxylated NPs, but more than the unmodified polystyrene. This might be due to differences in the morphology of these NMs, or to the higher density of positively charged groups on the amine-modified NPs than in the P(4VP)-MWNTs, which are only weakly positive [17]. A general observation is that SP-D binding to modified NMs is higher than to the unmodified NMs; thus a significant proportion of the SP-D was adsorbed from the cell free secretions, notably by 100 nm functionalized polystyrene NPs. This effect is perhaps not surprising since the modifications examined in this study relate to the addition of positive or negative charge and SP-D is a hydrophilic protein that, unlike all other surfactant

![Figure 4. SP-D adsorption to 50 nm unmodified, amine-modified and carboxyl-modified polystyrene latex NPs. (a) Representative immunoblots of 50 nm polystyrene NP-associated SP-D. (b) Data represented as bar graphs as the mean and s.e.m. from all experiments. The intensity of the SP-D signal was quantified by densitometry. Results are expressed as arbitrary units. Data represent mean ± s.e.m. from at least three experiments. Asterisks indicate a significant difference between treated samples and untreated control; *p < 0.05.](http://rstb.royalsocietypublishing.org/Downloaded from http://rstb.royalsocietypublishing.org/ on March 6, 2017)
proteins including SP-A, does not bind to surfactant phospholipids and is mostly soluble in alveolar fluid [26].

SP-D binding to 100 nm polystyrene was higher than to the 50 nm polystyrene. We expected more SP-D binding to the smaller, 50 nm spheres, reflecting increased surface area/unit mass than the 100 nm spheres. Clearly, SP-D adsorption was not necessarily a function of available surface area. However, even if the total amount of bound proteins was higher for the 50 nm NPs (which we have not measured) the behaviour of a single component of the protein corona is not predictable; it is likely that some proteins will bind more to the 50 nm polystyrene, while others will bind more to the 100 nm polystyrene [20]. In the case of SP-D, it exists as a dodecameric protein with a high molecular mass (greater than 540 kDa) and may undergo further multimerization to higher orders (approx. 1 MDa) and can therefore form a relatively large structure. We studied SP-D as it exists in lung secretions, rather than as purified SP-D; it is therefore possible that these larger isoforms were present. Together with the increased curvature of the smaller nanoparticles, binding of large complex molecules such as SP-D might be less feasible, and other components of the secretions might bind more tightly, to exclude SP-D.

As mentioned earlier, the human lung wash sample used in this study contains all the soluble proteins present in lung secretions, originating from a broad range of sources (e.g. mostly pulmonary cell products, but also serum), although it is depleted of the surfactant lipids and the surfactant-associated proteins SP-B, SP-C and SP-A. Thus, this model for studying SP-D binding to nanomaterials (NMs) better represents human lung lining liquid than studies utilizing purified SP-D preparations, because this model will take account of other proteins in the lung lining liquid and their impact (e.g. through competitive binding, concentration effects) on the propensity for SP-D to bind to nanomaterials, a situation that will exist in vivo. Similar studies utilizing purified SP-D will not have addressed this issue [8,9,12,13]. However, we acknowledge that in this study we have not taken account of the role of pulmonary surfactant in SP-D binding; binding of SP-A [7,12] or surfactant (phospho)lipids [4,10,27] may occur during translocation of NMs from the air through the surfactant layer before reaching the aqueous sub-phase. Clearly, this will be an important component of nanoparticle bioactivity in the alveolar unit.

SP-D binding to double-walled CNTs has been reported previously [9]; this study adds to the previous work, using MWNTs and additionally showing the importance of different surface functionalities in this process. Moreover, in this study we have used a wide concentration range of NMs to better reflect pulmonary exposure, which in the alveoli would normally be expected to be low; there was little effect of the NMs when used at 0.1, 0.5 and 1.0 μg ml⁻¹, although 5 μg ml⁻¹ AO-MWNTs caused a fourfold increase in SP-D adsorption into the pellet. Furthermore, at higher concentrations of all the MWNTs, as well as the polystyrene NPs, SP-D binding was significantly greater, indicating that chronic exposure, or inhalation of high concentrations of such NMs could be important in modifying the activity of SP-D, as well as altering the bioreactivity of the MWNTs [28]. The interaction of SP-D with latex NPs has also been reported [8]. The authors describe a change in size, charge and aggregation of NPs after addition of SP-D, measured by dynamic light scattering, zeta potential and scanning electron microscopy, which suggested surface attachment of the protein. They also suggest that other methods are required to identify the direct interaction between NPs and SP-D. This study, describing SP-D interaction with NPs using a different technique, that is SDS-PAGE and immunoblotting, confirmed their results and additionally shows the specificity of SP-D binding to NMs even in the presence of all the soluble protein secretions from peripheral human lung, contributing further to previous work in this area and indicates the significance of charge and size, as well as complexity of the milieu, in SP-D binding.

5. Conclusion

We have demonstrated that surface functionalization, concentration, charge and size of NMs affect their ability to adsorb SP-D from human lung wash. Negative surface charge enhanced SP-D binding, regardless of the class of NM. These results indicate the complexity of establishing the bioreactivity of nanoparticles at the alveolar gas–liquid interface and the importance of the first target, lung secretions and lung surfactant, in this process.

Ethics statement. Written informed consent was obtained for all lung tissue samples and the study was carried out with the approval of the Royal Brompton and Harefield Ethical Committee (Ref: 08/H0708/73).

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