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

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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

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carbohydrate recognition domain, the neck domain, a collag-
enateous domain and an N-terminal cysteine-rich domain. The
terminal carbohydrate recognition domain is important in
agglutination and eosinization of a variety of viruses, bacteria
and fungi. Moreover, it has been demonstrated that SP-D con-
tributes 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 inten-
tional 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 semi-
synthetic 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 recom-
binant 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 behav-
ior 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 pro-
teins 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 pulmon-
ary 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 adsor-
ation of SP-D from human lung secretions by NMs is
influenced by their physicochemistry, i.e. the material itself,
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 multi-
walled carbon nanotubes (MWNITs) 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 MWNITs (diameter 12.1 nm ± 3.7 nm, length 700 nm), syn-
thesized by chemical vapour deposition, were obtained from
Arkema SA (Lacq-Mourenx, France) and have been thoroughly
characterized [17]. The pristine, as-received MWNITs were function-
alyzed with poly(4-vinyl pyridine) (P(4VP)-MWNIT) and acid
oxidized (AO-MWNIT) to provide three surface modifications
[17]. Pristine MWNITs are weakly acidic with an isoelectric point
of 4.9; the P(4VP)-MWNITs have a nitrogen-containing group,
shifting the isoelectric point to 7.0; acid oxidized AO-MWNITs pre-
sent a generally acidic surface, with a complex mixture of
functional groups present [17]. Prior to incubation with human
lung secretions, MWNITs were suspended in PBS at a concen-
tration 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 poly-
styrene 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 carcino-
ma 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 macro-
phages. The supernatants were analysed for total protein using
the Bradford assay solution to give a working volume of 200 μl.
The optical density was measured at 570 nm and protein concen-
tration was calculated from regression data generated from a
BSA standard curve. The fractions with the highest protein con-
tent 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 supernatant [18]. SDS-
PAGE and immunoblotting (described in §2e) 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 supernatant 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 supernatant was incubated with
the same volume of increasing concentrations of MWNITs

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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-VP)-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)]. It was only possible to reveal a significant decrease in the supernatant SP-D content when adsorption of SP-D to the corresponding NM was particularly high, usually at the greatest NM concentrations. This might reflect relatively high levels of SP-D originally present in the supernatant fraction, as well as the fact that immunoblotting is not sufficiently sensitive to reveal a slight decrease of SP-D level in such a sample.

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
Figure 2. SP-D adsorption to pristine, P(4VP)-modified and acid oxidized MWNTs. (a) Representative immunoblots of MWNT-associated SP-D. (b) Data represented as bar graphs as the mean and SEM 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, **p < 0.01.

Figure 3. SP-D adsorption to 100 nm unmodified, amine-modified and carboxyl-modified polystyrene latex NPs. (a) Representative immunoblots of 100 nm polystyrene NP-associated SP-D in row (P); row (S) shows the SP-D remaining in the supernatant. (b) The upper row shows the non-adsorbed SP-D remaining in the supernatant (S). The lower row shows the NP-associated SP-D (P). 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 < 0.01, ***p < 0.001.

non-particle control at a particle concentration of 40 µg ml⁻¹ (figure 4). There was no change in SP-D level in the supernatant fractions following treatment with the 50 nm polystyrene NPs (data not shown).

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 defence from respiratory pathogens, there might be a higher susceptibility to microbial
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 agglomeration; 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)-MWN Ts. 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-MWN Ts. Since all the MWN Ts were of the same length and diameter, the strong binding of SP-D to AO-MWN Ts 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-MWN Ts 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 × 10⁷ 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 × 10⁶ 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 MWN Ts, 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.
proteins including SP-A, does not bind to surfactant phospholipids and is mostly soluble in alveolar fluid [26]. SP-D binding to 100 nm poly styrene 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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References


4. Bakshi MS, Zhao L, Smith R, Possmayer F, Petersen MZ. A.P. acknowledges an individual ERC starting grant for additional funding for S.C. (project number: 257182). This work was carried out as part of the MRC-PHE Centre for Environment and Health, supported jointly by the Medical Research Council and Public Health England. This work was also supported by the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London.


