Inhaled nanoparticles develop a complex protein corona: biological importance and implications for sustained release formulations

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SUMMARY

Nanoparticle delivery systems are being designed as carriers for targeting drugs to the lungs, but when they deposit into lung lining fluid they are surface-modified by interaction with proteins and lipids. To date there is little information on the biomolecular corona that forms around nanoparticles in the lungs. Here we identify the protein corona that forms rapidly around nanoparticles in human lung lining fluid. Nanoparticles (silicon dioxide and polyvinyl acetate) were incubated for 1 h in human lung lining fluid and the hard corona was isolated. The proteins adhered to the particle surface were extracted and run through a 1-D SDS-gel. The bands of interest were cut and the proteins were extracted using trypsin digestion prior to injecting into the LC-MS/MS. Comparison with the Uniprot-SwissProt Human proteome database revealed enrichment around the particle surface of surfactant proteins, complement factors, immunoglobulins and other proteins with profound immunological actions, such as complement components. Surfactant proteins act as innate immunity proteins and opsonise microbes and particles and promote their clearance by phagocytosis by alveolar macrophages. Surfactant protein A coating has been shown to enhance the uptake of metal oxide nanoparticles as compared to metal oxide nanoparticles coated with albumin (the major plasma protein). This data improves our understanding of the behaviour of inhaled nanoparticles and informs the design of safe and effective nanomedicines such as those that aim to control the release of drugs in the lungs.

INTRODUCTION

Drug particles from current orally inhaled products present a continuously dissolving surface, whereas insoluble particles or carriers designed for sustained drug release in the lungs present a surface to which adsorption can occur. Inhaled nanomedicines, which have a particularly large surface area to mass ratio, have been advocated as particularly promising as a controlled release technology[1,2]. Although the interaction of nanoparticles with the body is considered to be a product of their small size and large surface area to mass ratio, the mechanisms that govern the fate and effects of inhaled nanoparticles are not fully understood. It has been demonstrated that when nanoparticles are introduced into a biological environment, a protein corona forms[3]. For example, nanoparticles adsorb an abundance of plasma proteins which form a biomolecular corona, the importance of which has been reviewed recently by Monopoli *et al.* (2012)[4]. This corona has been shown to confer biological effects – for example, to decrease cellular uptake of silica nanoparticles[5], to impair the targeting capabilities of transferrin-functionalised silica nanoparticles[6] and reduce the cytotoxicity of cationic nanoparticles[7]. Thus, the corona interface forges the biological identity of the particle with a clear impact on the interaction of particles with cells.

Human lung lining fluid differs from human plasma compositionally in terms of the abundance of certain proteins, the presence of surfactant proteins and antioxidants. Whilst employing plasma has been useful for proof-of-concept studies, the differences between the proteome and lipid profile of plasma and lung lining fluid will result in significant differences in the biomolecular coronae of nanoparticles in the two compartments. The aim of this study was to identify for the first time the biomolecular protein corona formed around the nanoparticles when they come in contact with human lung lining fluid.

MATERIALS AND METHODS

BAL Sample Collection and Proteomic Characterization

Parallel broncho-alveolar lavages and plasma samples were obtained from healthy (n=16, 25.0±2.6 years, 6M/10F) subjects. Peripheral blood was collected to determine plasma ascorbate and urate concentrations. Broncho-alveolar lavage (3 x 60 ml) was performed after wedging the scope in the right middle lobe / left lingula and each sample was treated with 50% metaphosphoric acid immediately after collection.

Nanoparticle Sample Preparation
Two different nanoparticle types, polyvinyl acetate (PVAc) and silicon dioxide (SiO_2), were incubated in human lung lining fluid to match the surface area to protein ratio used by Monopoli et al.[8] for 10% plasma concentration. The surface area to protein ratio was kept constant for both the particle types. NPs were allowed to incubate with the lung lining fluid solutions on ice for one hour. To obtain hard protein corona complexes, the samples were centrifuged to pellet the particle-protein complexes and separated from the supernatant. The pellet was then resuspended in 500 µl of PBS and centrifuged again 1 h at 20,000 *g* at 4°C. These washing-steps before resuspension of the final pellet in 14 µl PBS removed proteins with low affinity for the NP surface (the soft protein corona).

SDS-PAGE & Protein Corona Identification by Mass Spectrometry
Immediately after resuspension of the nanoparticle/protein corona pellet, 7 µl of protein loading buffer [62.5 mM Tris-HCL pH 6.8, 2% (w/v) SDS, 10% glycerol, DTT and 0.04 M 0.01% (w/v) bromophenol blue] was added. The sample was heated for 5 min at 100°C, then loaded on a 12% gel polyacrylamide gel. Gel electrophoresis was performed at 120 V, 400 mA for about 30 min until the proteins entered the separation gel. The gels were stained for one hour in Coomassie blue [50% methanol, 10% acetic acid, 2.5% (w/v) brilliant blue]. Bands of interest from SDS-PAGE gels were excised and digested in gel with trypsin according to the method of Shevchenko et al[9]. The resulting peptide mixtures were re-suspended in 0.1% formic acid and analyzed by electrospray liquid chromatography mass spectrometry (LC-MS/MS) using Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer Chromatography solutions (Buffer A, 0.1% formic acid; Buffer B, 100% acetonitrile and 0.1% formic acid) were run as a 72 min gradient with a flow rate of 150 µl/min as the electrospray source.

Criteria for Protein Identification
Scaffold (version Scaffold_3.6.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm[10]. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides.

RESULTS AND DISCUSSION

BAL Proteomic Characterization
Using LC-MS/MS more than 200 unique proteins were classified according to their gene ontological definition. The most abundant proteins were serotransferrin, immunoglobulin, serum albumin, and alpha-1-antitrypsin which are also abundant plasma proteins (table 1). Lung specific proteins quantified included surfactant protein A, B & D and clara cell secretory protein 16 (CC16). The ontological classification of the extracellular proteins identified showed that 45% had immune or inflammatory function (Figure 1a).

Protein Corona Identification
The hard corona was isolated from PVA and SiO_2 nanoparticles and semi-quantitative determination of the composition of hard corona was performed by LC-MS/MS. More than 500 unique proteins were identified in the biomolecular corona formed around the particles. The most abundant proteins identified in the PVA corona were surfactant protein A, serum albumin, complement C3, apolipoprotein A-1 and surfactant protein B whilst the most abundant proteins identified in the SiO_2 corona were surfactant protein A (SP-A), serum albumin, complement C3, surfactant protein B (SP-B) and complement C4 (Table 1). Overall, the protein corona of PVA and SiO_2 were similar with more than 400 common proteins identified. The ontological classification of the extracellular proteins in the biomolecular corona formed around the PVAc and SiO_2 particles showed that majority of the proteins identified (>50%) had immune and inflammatory function (Fig. 1b and 1c).

Table 1. The ten most abundant proteins identified in BAL and biomolecular corona around PVAc and SiO_2 nanoparticles using LC-MS/MS. The data represents mean intensity obtained from n=3 experiments.

BAL		PVAc		SiO_2	
Protein	Intensity (AU 10^9)	Protein	Intensity (AU 10^9)	Protein	Intensity (AU 10^9)
Serotransferrin	13	SP-A	2.8	SP-A	2.5
Ig gamma-1 chain	12	Serum Albumin	0.75	Serum Albumin	1.3
Ig gamma-3 chain	9.4	Complement C3	0.61	Complement C3	0.81
Ig gamma-2 chain	9.1	Apolipoprotein A-I	0.29	SP-B	0.59
Ig gamma-4 chain	9.1	SP-B	0.28	Complement C4	0.30
Ig kappa chain	5.9	Alpha-1-antitrypsin	0.26	Alpha-1-antitrypsin	0.28
Protein C3orf38	4.1	Fibronectin	0.24	Apolipoprotein A-I	0.24
Serum albumin	4.0	Complement C4	0.2	Actin	0.22
Ig lambda-2 chain	3.1	Ig alpha-1	0.19	Ig gamma-1	0.16
Alpha-1-antitrypsin	2.7	Annexin A2	0.17	Fibronectin	0.15
>200 proteins identified	$>1x10^6$	>500 proteins identified	$> 1x10^6$	>450 proteins identified	$>1x10^6$

We observed an approximate 13-fold enrichment of SP-A on both the particles, an 11-fold enrichment of SP-B on PVAc and 23-fold enrichment of SP-B on SiO_2 but no enrichment of surfactant protein D (SP-D) on either of the particle surface (Figure 2). Proteins of the complement system were found to be significantly enriched on the particle surface, with complement C3, C1q, C4-A and C2 enriched approximately 50-, 30-, 12- and 4-fold, respectively. Furthermore we also found the enrichment of Apolipoprotein A-IV and A-I by 8- and 2.5-fold, respectively.

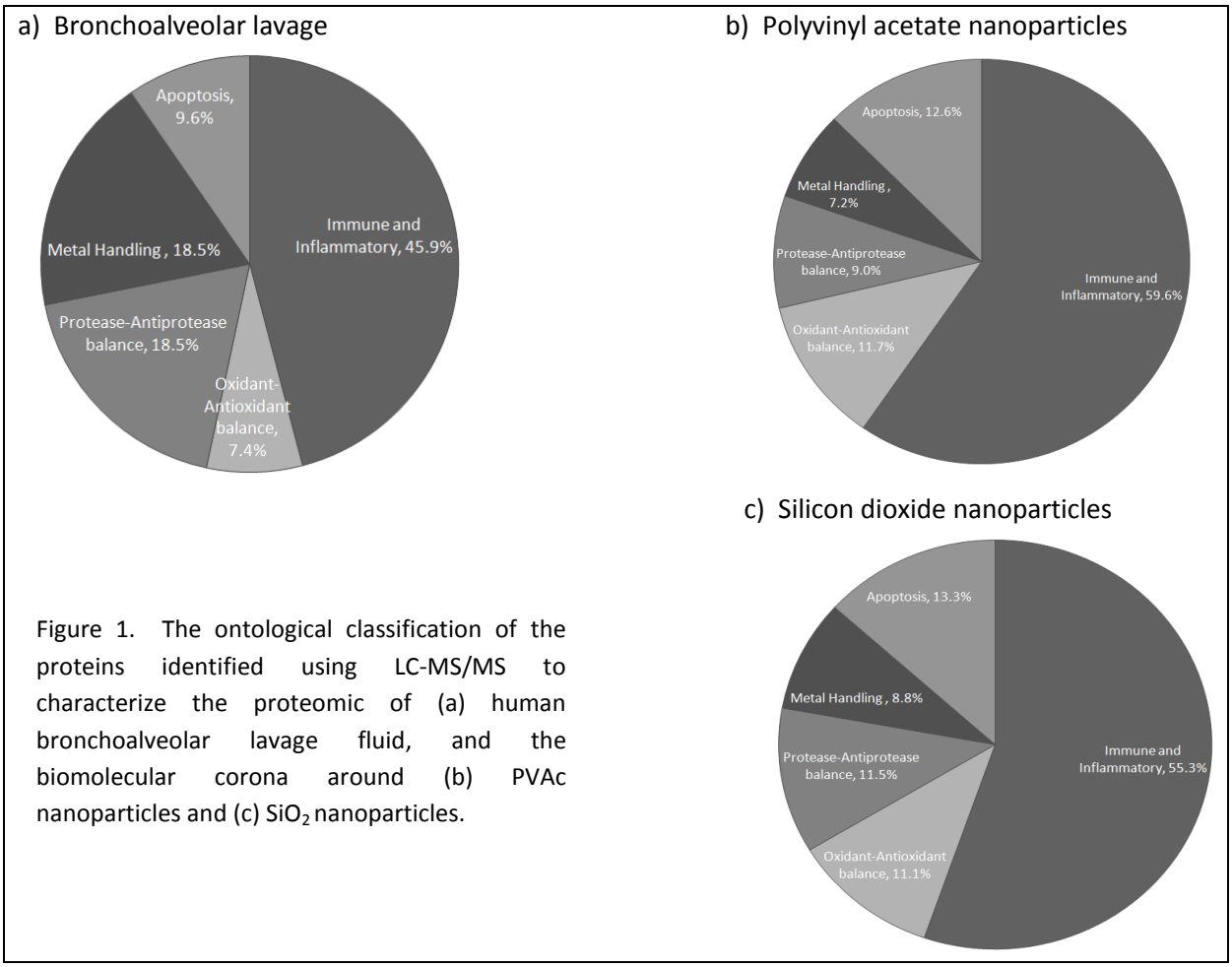

Figure 1. The ontological classification of the proteins identified using LC-MS/MS to characterize the proteomic of (a) human bronchoalveolar lavage fluid, and the biomolecular corona around (b) PVAc nanoparticles and (c) SiO_2 nanoparticles.

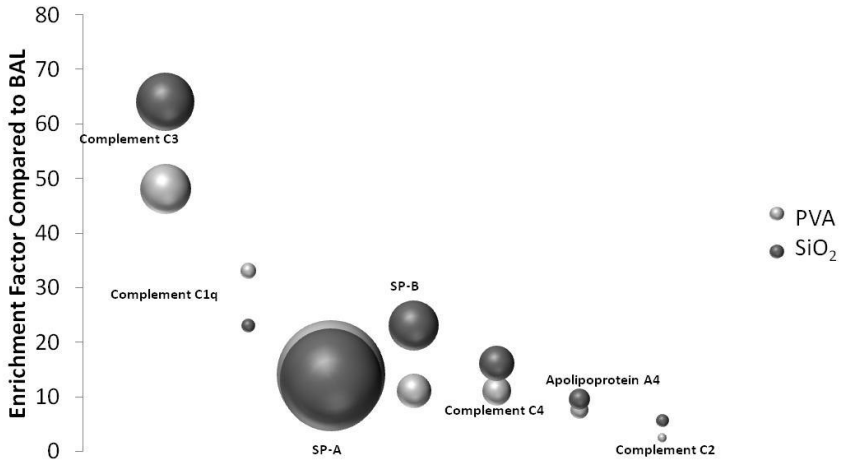

Figure 2: Protein enrichment at the surface of PVAc (light grey) and SiO_2 (dark grey) nanoparticles compared to

bronchoalveolar lavage fluid and the abundance of the proteins (represented as the size of the bubble). Enrichment factor is unit less and represents the ratio of intensity of the protein adhered to the nanoparticle to intensity of the same protein in BAL.

The composition of the protein corona of nanoparticles will profoundly affect the lung after nanoparticle exposure and the enrichment of surfactant on inhaled nanoparticles will have biological implications. The formation of a biological bilayer around particles is immediate (less than 2 mins) as shown Mornet *et al.* using cryo-electron microscopy[11]. Surfactants SP-B and surfactant protein C (SP-C) are predominantly involved in decreasing surface tension and keeping the airways and alveoli expanded during exhalation. However, surfactant also acts as a barrier and contributes to innate immune defence. SP-A and SP-D attach to inhaled particles and microbes to opsonise them and promote their clearance by alveolar macrophage phagocytosis. The absence of SP-D has been shown to decrease the uptake of polystyrene particles in primary alveolar macrophages and dendritic cells[12]. Activated complement components are also important in particle opsonisation (similar to surfactant proteins) and have active effects in a range of other immune and inflammatory pathways. Particles enrich sufficient complement components to activate complement convertases that generate active complement mediators such as C3a and C3b. Pham *et al.*[13,14] have previously shown that perfluorocarbon lipid-encapsulated nanoparticles can activate the complement pathway. Our research shows that interaction with complement components is likely a generic consequence upon deposition of nanoparticles in lung lining fluid. The biological relevance of particulate-activated complement has been shown by Walters *et al*.[15] – in a murine model of ambient particulate matter-induced pulmonary responses they showed C3 to be a mediator of nanoparticle-induced airways hyper-responsiveness.

CONCLUSIONS

The importance of using human lung lining fluid to develop a better understanding of the interaction of inhaled particles and airway cells *in vitro* has been highlighted[16]. We have elucidated for the first time the protein corona profile of nanoparticles in the human lung lining fluid, identifying the enrichment of surfactant and complement proteins. This is critical for developing a better understanding of the nano-biological interface in the human lungs and informs the design of safe and effective inhaled nanomedicines for the next generation of drug delivery to the lungs.

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