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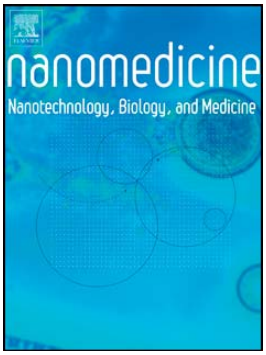

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Differences in the coronal proteome acquired by particles depositing in the lungs of asthmatic versus healthy humans

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Abstract

Most inhaled nanomedicines in development are for the treatment of lung disease, yet little is known about their interaction with the respiratory tract lining fluids (RTLF). Here we combined the use of nano-silica, as a protein concentrator, with label-free snapshot proteomics (LC-MS/MS; key findings confirmed by ELISA) to generate a quantitative profile of the RTLF proteome and provided insight into the evolved corona; information that may be used in future to improve drug targeting to the lungs by inhaled medicines. The asthmatic coronal proteome displayed a reduced contribution of surfactant proteins (SP-A and B) and a higher contribution of α1-antitrypsin. Pathway analysis suggested that asthmatic RTLFs may also be deficient in proteins related to metal handling (e.g. lactoferrin). This study demonstrates how the composition of the corona acquired by inhaled nanoparticles is modified in asthma and suggests depressed mucosal immunity even in mild airway disease.

Short Communication

Recent studies have shown how inhaled particle surfaces are modified when immersed in respiratory tract lining fluid (RTLF), becoming enriched with immune regulatory proteins, forming a corona that will be presented to the epithelium or phagocytic cells at the mucosal surface (1–4). This corona masks the original particle surface, changing its biological identity and potentially modifying the nature of the particles' interaction with the lungs, e.g. toxicity, cellular uptake and, in the case of nanomedicines, therapeutic efficacy (2). An understanding of these interactions is therefore essential for the development of inhaled medicines and yet in contrast to the abundance of published work on intestinal fluids and their interactions with oral dosage forms in the field of oral biopharmaceutics (5), little work has focused on particle interactions with RTLF components, especially in the context of target lung diseases (1). In order to address this knowledge gap here we report the first visualization the corona formed in human RTLF and provided a detailed characterization of the biomolecular corona of particles in healthy versus asthmatic RTLFs.

We studied the corona formed around silica nanoparticles (200 nm), a well characterized analytical standard (1), incubated in concentrated bronchoalveolar lavage (BAL) fluids obtained from atopic asthmatics (PC_{20} <8 mg/mL methacholine, treated with short-acting inhaled β2-agonists on demand) and healthy control subjects. For these studies we employed lavage fluids from 5 healthy volunteers (26.0 ± 2 years, 2M/3F) and 5 asthmatics (25 ± 6 years, 1M/4F, PC_{20}=3.1 ± 2.6) concentrated back to their undiluted RTLF levels based on the ratio of lavage to plasma urea concentrations (a dilution factor to indicate the degree to which RTLF has been diluted in the recovered BAL fluid (6)), prior to pooling to provide a generic healthy and asthmatic sample. Experimental details are outlined in the supplementary material. It was important to control for age as a factor because of the known age-related changes in the lung, reflected functionally by decreased lung volumes, reduced mucociliary clearance and increased susceptibility to infection (7).

Cryogenic transmission electron microscopy was employed to provide a direct visualization of corona formation around nanoscale particles after incubation in human RTLF from a healthy volunteer (**Figure 1A**). Electron micrographs revealed a clearly visible corona, appearing as a continuous layer of electron-dense coating with a thickness of 3-5 nm surrounding the particles.

This is in agreement with similar observations of corona formation around silica nanoparticles using Survanta®, an FDA approved bovine lung surfactant (1). The albumin-depleted coronal proteome was characterised by label-free quantitative liquid chromatography mass spectrometry (LC-MS/MS) using established methods (1), with over 400 coronal proteins identified, of which the most abundant 20 are highlighted in **Table 1**. For reasons of analytical sensitivity, it was necessary to deplete the lavage concentrates of albumin before incubation of the particles and proteomic characterization. Thus, the contribution of the most abundant RTLF protein to the corona is artificially under-represented in the analysis. We did not address the lipid components of the corona, although these are thought to be less discriminating (3, 4).

Identified proteins were functionally classified using SwissProt and NCBI databases (**Figure 1B**), with the abundance of individual proteins expressed as the percentage of the intensity of all the proteins identified. Using this approach a ~10% decrease in the contribution of innate immunity proteins was identified in the asthmatic corona, with surfactant protein A (SP-A) the most dysregulated (0.47-fold decrease; **Figure 1B*i***). A 5% decrease in the lipid metabolism/transport proteins was also observed in the asthmatic corona (**Figure 1B*ii***). Conversely, the asthmatic corona was associated with a 5% increase in proteins involved in protease-antiprotease activity (**Figure 1B*iii***), including a 1.7-fold increase of α1-antitrypsin. These differences in the coronal proteome reflect differences reported previously in the composition of BAL fluid from asthmatics. Wu *et al.* (8) and Cederfur *et al.* (9), using LC-MS/MS, also found that the majority of the differentially expressed proteins in asthmatic RTLFs were associated with immune response (23%), lipid metabolism (12%) and proteolysis (9%). Thus, the coronal proteome appears to represent a concentrated fingerprint reflecting underlying protein composition of the host RTLF (10). Differences between the corona formed in healthy and asthmatic RTLF were evaluated further using iPathwayGuide analysis to obtain biological insights by identifying the pathways/functions most impacted by the observed changes between the two groups. This software analysis tool implements an 'impact analysis' approach, which takes into consideration not only the number of differentially expressed genes, or in this case proteins (i.e. enrichment analysis), but also topological information such as the direction and type of all signals in a pathway, and the position, role, and type of each protein (11). Following quantile normalization and Benjamini–Hochberg correction for false discovery rate, we identified 26 differentially adhered proteins in the asthmatic corona (**Figure 1C**). Pathway analysis of these proteins demonstrated they were involved in immune system processes (**Figure 1C*i***) and metal ion binding (**Figure 1C*ii***), with lactoferrin contributing to both pathways.

To evaluate the accuracy of this semi-quantification of coronal protein content, selected proteins were quantified by ELISA in lavage samples, both bronchial wash (BW) and BAL fluid from an expanded group of asthmatics (26.0 ± 6 years, 6M/10F, PC_{20}=2.5 ± 2.6) and healthy (25 ± 2 years, 11M/5F) subjects. Three proteins were examined Immunogobulin A (IgA, the major mucosal immunoglobulin, the levels of which appeared equivalent in the proteomic analysis), surfactant protein A (SPA, 0.47-fold decreased in asthmatics) and alpha-1-antitrypsin (α1AT, 1.74 fold increased in asthmatics) (Table 2). Although the differences in protein concentrations as determined by ELISA analysis did not attain statistical significance, the observed trends for reduced SP-A (P=0.06) and increased alpha-1-antitrypsin were consistent with the proteomic analysis. We extended the validation panel to include five further proteins, 4 selected of the basis of their high abundance within the coronal proteome (albumin, IgG, IgM and transferrin), and

the fifth due to the evidence of suppressed innate immune defense proteins (lysozyme). These data are summarized in Supplementary Table 1, together with the predicted fold changes obtained from the proteomic analysis. Of these proteins both transferrin and lysozyme appeared to most depressed within the mild asthmatic corona, 0.16 and 0.34-fold respectively. The more comprehensive ELISA data within the BAL fluid did not confirm this finding, but a significant ($P<0.05$) decrease in transferrin within the asthmatic BW samples was observed, with a trend ($P=0.06$) toward decreased lysozyme concentrations also within the proximal airway sample.
Whilst the proteomic and ELISA results were not wholly concordant, these data overall, together with the pathway analysis, could be suggestive of depressed innate immune defense in mild asthmatics. Since the deficiencies in immune proteins identified within the asthmatic RTLF may increase susceptibility to microbial infection (12), lipopolysaccharide (LPS) levels were quantified to provide a proxy measure of bacterial load within the airway (see supplementary material). BAL fluid LPS activity was found to be 1.98-fold higher in asthmatics compared to healthy subjects, but did not attain statistical significance (Table 2). In contrast, a significant 6.6-fold increase in LPS was noted in the more proximal BW sample from the asthmatics ($P<0.05$).

These data provide a detailed molecular characterization of the asthmatic coronal proteome, with evidence of deficiencies in proteins involved in innate immunity, lipid metabolism/transport and metal handling. Our observations may raise concerns about the impact of proposed therapeutic inhaled nanomedicines on innate immunity in the lung. The mechanism we have employed here to concentrate RTLF proteins, for characterisation purposes, may *in vivo* result in an unwanted sequestration of RTLF proteins. Axiomatically, the adsorption of proteins that trigger phagocytosis of a particle provides a clearance-promoting mechanism. SP-A binding has been shown to trigger the uptake of particles in murine alveolar macrophages (13, 14), thus its reduced presence in the asthmatic corona may impair bacterial clearance. Similarly, the formation of the particle corona may lead to local depletion of innate immunity proteins at the surface of the lungs. Thus whilst the formation of the corona may modify particle clearance (15–17) thereby affecting drug disposition in the lungs, it may also increase the susceptibility of the airway to infection. Consistent with this, exposure to ultrafine carbon black particles has been shown to increase the susceptibility of the lung to both bacterial (18) and viral infections (19) *in vivo*. Susceptibility to infection has also been reported in populations exposed to cigarette smoke (20) combustion-derived air pollution (21) and occupational aerosols (22), This therefore raises the question as to whether inhaled slowly disintegrating nanoscale drug formulations may have adverse effects on immunity in the lung, especially with regular use. Such considerations do not apply to current inhaled medicines which exist transiently, if at all, in solid particle form in RTLF, although interestingly studies have demonstrated an increased risk of pneumonia in COPD patients using poorly soluble inhaled corticosteroids (23, 24). It is clearly important to understand particle-RTLF interactions and further work is needed in this area focused on relevant patient groups.

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Table 1: The 20 most abundant proteins (in rank order) based on the mean intensity of the three major precursor ions identified in the SiO_2 corona evolved after a 1 hour incubation in concentrated lavage pooled from healthy and asthmatic subjects. For each protein in the healthy corona, the ranking in terms of abundancy in the asthmatic group is provided (and vice versa). Albumin is highlighted as the signal reflects the residual contribution after pre-depletion of albumin (the most abundant protein in RTLF) to enable identification of other proteins.

Top 20 abundant proteins in the SiO_2 corona					
Healthy Controls			**Mild asthmatics**		
Identified Proteins	**Relative rank in asthmatics**	**Molecular Weight**	**Identified Proteins**	**Relative rank in healthy controls**	**Molecular Weight**
Pulmonary surfactant-associated protein A2	1	26 kDa	Pulmonary surfactant-associated protein A2	1	26 kDa
Serum albumin	2	69 kDa	Serum albumin	2	69 kDa
Pulmonary surfactant-associated protein B	6	42 kDa	Alpha-1-antitrypsin	6	47 kDa
Apolipoprotein A-I	9	31 kDa	Actin, cytoplasmic 1	5	42 kDa
Actin, cytoplasmic 1	4	42 kDa	Ig alpha-1 chain C region	8	38 kDa
Alpha-1-antitrypsin	3	47 kDa	Pulmonary surfactant-associated protein B	3	42 kDa
Complement C3	7	187 kDa	Complement C3	7	187 kDa
Ig alpha-1 chain C region	5	38 kDa	Complement C4-B	9	193 kDa
Complement C4-B	8	193 kDa	Apolipoprotein A-I	4	31 kDa
Hemoglobin subunit beta	12	16 kDa	Ig gamma-1 chain C region	11	36 kDa
Ig gamma-1 chain C region	10	36 kDa	Polymeric immunoglobulin receptor	14	83 kDa
Ig kappa chain C region	33	12 kDa	Hemoglobin subunit beta	10	16 kDa
Ubiquitin-60S ribosomal protein L40	63	15 kDa	Fibronectin	22	263 kDa
Polymeric immunoglobulin receptor	11	83 kDa	BPI fold-containing family B member 1	17	52 kDa
Annexin A2	32	39 kDa	Napsin-A	16	45 kDa
Napsin-A	15	45 kDa	Vitamin D-binding protein	56	53 kDa
BPI fold-containing family B member 1	14	52 kDa	Alpha-enolase	18	47 kDa
Alpha-enolase	17	47 kDa	Hemoglobin subunit alpha	45	15 kDa
Apolipoprotein A-IV	34	45 kDa	Ig mu chain C region	27	49 kDa
Ig gamma-3 chain C region	30	41 kDa	Glutathione S-transferase A2	94	26 kDa

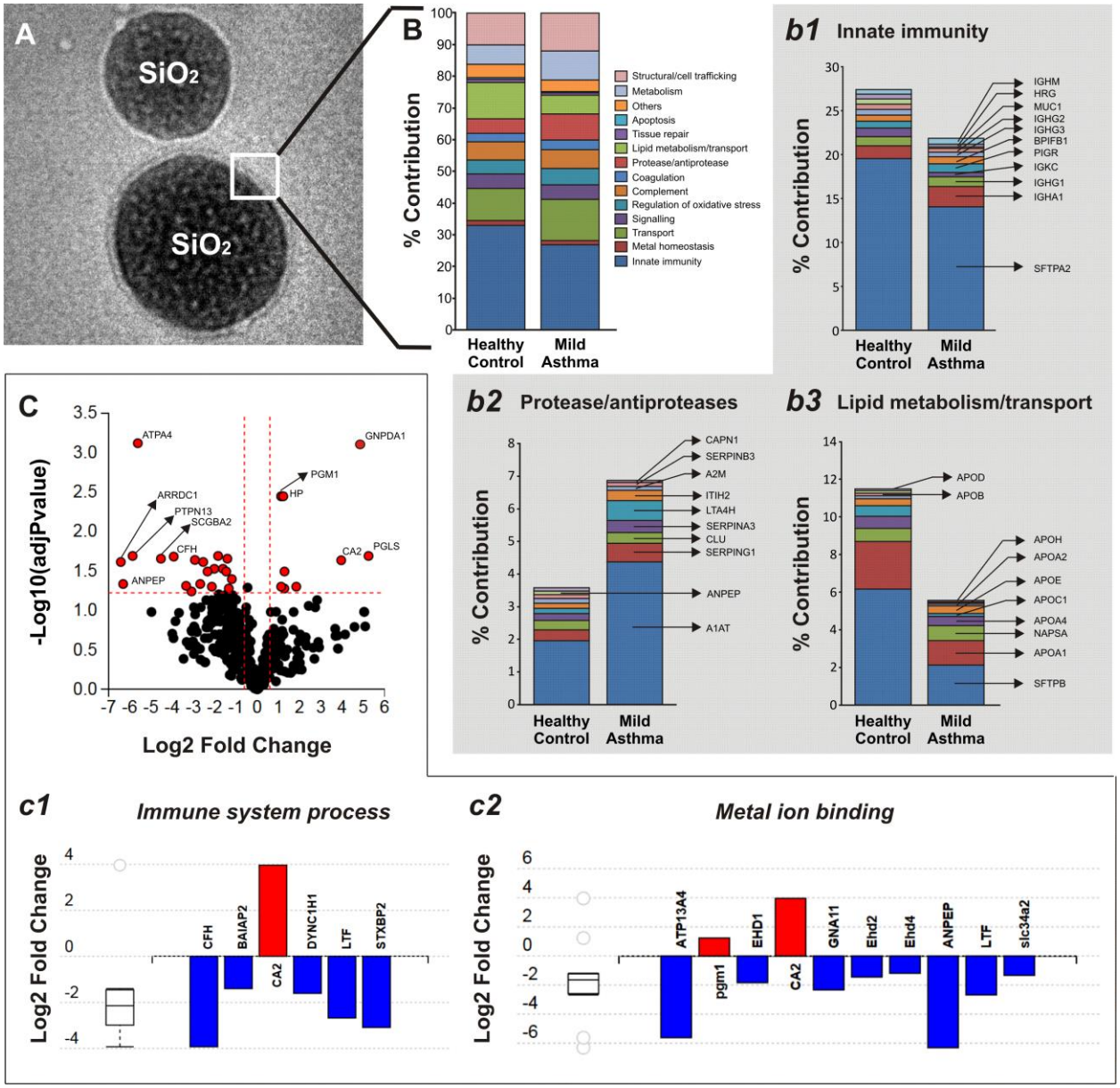


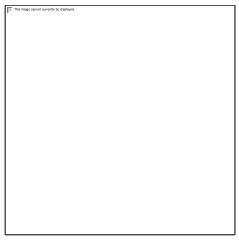
Figure 1: Proteomic characterization of the hard corona formed around SiO_2 particles incubated in concentrated bronchoalveolar lavage (BAL) fluids from healthy and mild asthmatic subjects. Panel A, cryoTEM image of SiO_2 particle incubated in concentrated healthy human BAL fluid (see Supplementary Information Figure 1).
Panel B, functional classification and relative abundance of proteins identified in the healthy and asthmatic corona; panel *b1*, most abundant innate immunity proteins within the corona of the two groups (proteins are identified using their gene IDs); panel *b2*, most abundant proteins in the protease/antiprotease group; panel *b3*, relative contribution of proteins involved in lipid metabolism and transport.
Panel C illustrates the results of the iPathway analysis, with proteins with significant over, or under representation in the asthmatic corona (relative to the healthy; see Supplementary Information Figure 2) highlighted using a volcano plot; panel *c1*, illustrates perturbation of

proteins within the asthmatic corona related to immune system processes, using the conventional GO ontology; and panel *c2*, perturbations in proteins related to metal ion binding.

Table 2: Selected protein concentrations and LPS activity quantified in bronchial wash and bronchoalveolar lavage fluid samples from expanded groups of healthy and asthmatic subjects. Data represent median values with the 25th and 75th percentiles, n=14-15 per group, reflecting the availability of lavage material.

		Bronchial wash		**Bronchoalveolar lavage**	
	Fold change (Proteomic analysis)	Healthy	Mild asthmatic	Healthy	Mild asthmatic
SP-A (mg/ml)	0.47	1.04 (0.60-1.54)	0.46 (0.23-0.70)	1.00 (0.61-1.31)	0.64 (0.46-0.87)
α1AT (mg/ml)	1.74	0.68 (0.39-1.09)	0.38 (0.26-0.44)	0.94 (0.55-2.16)	1.55 (1.35-2.19)
IgA (mg/ml)	1.11	4.93 (1.80-7.76)	3.68 (0.67-4.92)	0.72 (0.53-0.97)	0.65 (0.28-1.24)
LPS (EU/ml)[A]	NA	4.1 (1.6-13.3)	27.1 (8.7-125.9)*	14.3 (5.5-40.4)	28.3 (9.2-76.0)

All protein concentrations and LPS activities have been adjusted for sample dilution based on the ratio of plasma to lavage urea concentrations. Comparison of healthy and mild asthmatic lavage protein concentrations was performed using the Mann-Whitney U test, with significance assumed at the 5% level. A: Lipopolysaccharide activities were determined using Limulus Amebocyte Lysate assay and reported in EU/ml.



Graphical Abstract

The composition of the corona acquired by inhaled nanoparticles is modified in asthma and suggests depressed mucosal immunity even in mild airway disease