Development of a Targeted Mass Spectrometry Protein Assay to Identify Early Stages of Pulmonary Response to Carbon Nanotube Exposure in a 3D Lung Model

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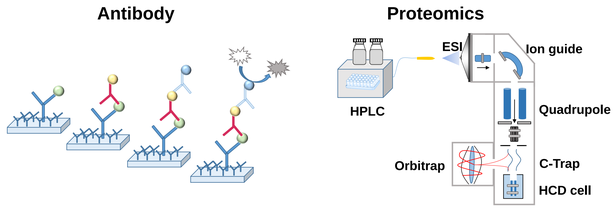
INTRODUCTION

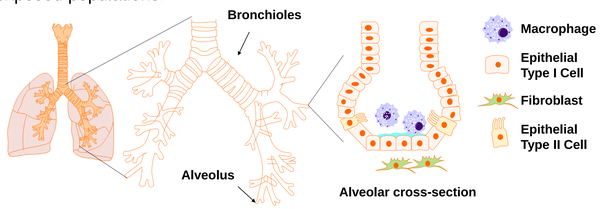
Over the past 10 years, liquid chromatography tandem mass spectrometry (LC-MS/MS) based proteomics has been increasingly used for protein assay development owning to its promise for specific, reproducible, and quantitative measurement of changes in protein expression levels.[1] Proteomics offers advantages over monoclonal antibodies by measuring proteins without the use of animal-derived materials and providing a higher throughput measurement of 1000s of proteins per sample.



Proteins = 1/sample (96)
Data points per assay = 96

Proteins = 2500/sample (48)
Data points per assay= 120,000

The study described herein demonstrates the use of global proteomics to thoroughly investigate the cellular response of multi-walled carbon nanotube (MWCNT) exposure using an *in vitro* tri-culture lung model. MWCNTs possess fiber-like characteristics that are similar to asbestos, thus creating concern for possible induction of pulmonary fibrosis in exposed populations.[2,3]



PROTEOMIC WORKFLOW

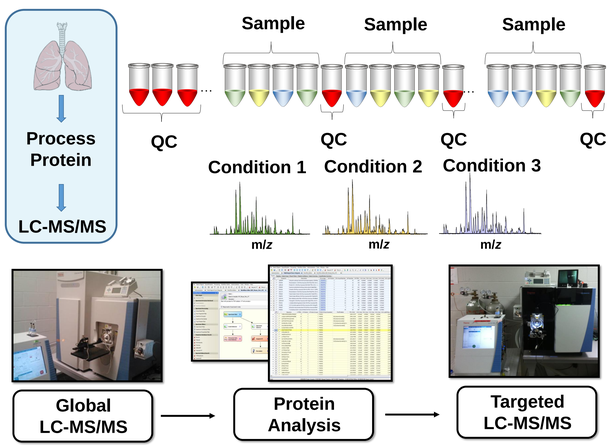


Figure 1: Proteomic sample preparation overview. All samples were processed on the following ThermoScientific LC-MS/MS instruments: Q-Exactive Plus (Discovery) and Quantiva (Targeted).[4,5]

CELL CULTURE AND EXPOSURE METHOD

CULTURE MODEL

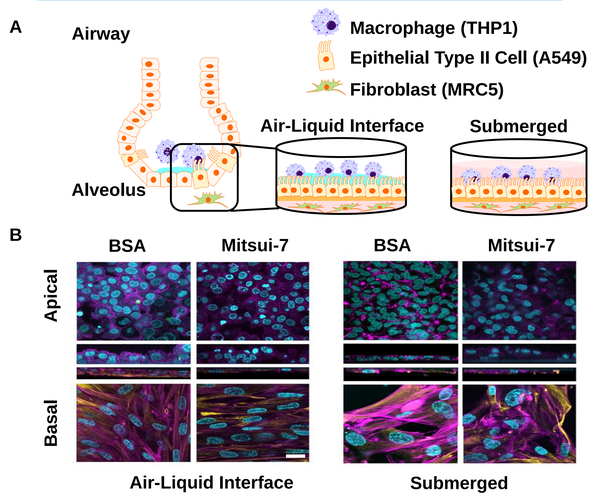


Figure 2: (A) Air-Liquid Interface (ALI) and suspended Mitsui-7 exposure. **(B)** Cell morphology: Apical, basolateral, and cross-section images were collected at 96h. F-actin is shown in magenta, the cell nuclei in cyan, and the intermediate filaments on the basolateral side in yellow. Scale 20 µm.

MWCNT EXPOSURE METHOD

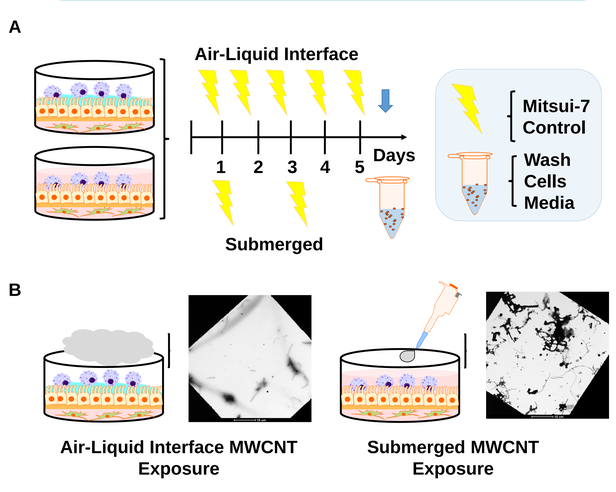


Figure 3: (A) Timeline for Mitsui-7 (M-7) exposure and collection. Submerged total exposure dose equals 18 µg/mL. ALI exposure deposition equals 200 ng/cm^2 of bovine serum albumin (BSA)/day and 2 µg/cm^2 of Mitsui-7/day (10 µg/cm^2 total). **(B)** TEM images of Mitsui-7 suspensions for ALI and Submerged exposure method.[6]

DATA ANALYSIS

ANOVA MODEL

Protein Intensity = Intercept + Exposure Method + Material + Error

	Df	Sum Sq	Mean Sq	F value	P value
Exposure Method	**1**	**5.466e+18**	**5.466e+18**	**19.837**	**<0.0001**
Material	**1**	**1.595e+17**	**1.595e+17**	**0.579**	**0.4468**
Residuals	**2114**	**5.899e+20**			

Figure 4: ANOVA model was fit using the media sample data sets.

PROTEIN LEVEL ANALYSIS

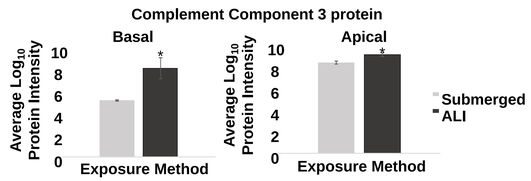


Figure 5: Example comparison analysis used to evaluate significance on the protein level (3 replicates/group). Welch t-test was used to calculate all *p*-values.

ENRICHMENT ANALYSIS

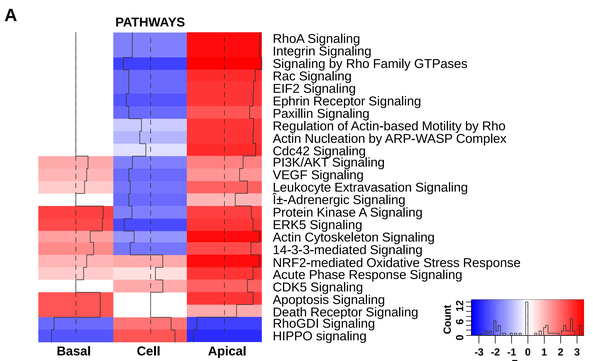


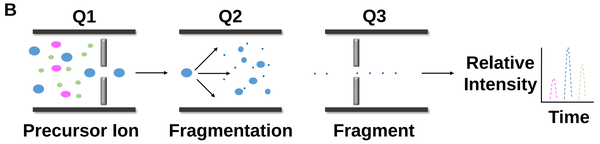
Figure 6: Heat maps show top 25 enriched **A)** Pathways and **B)** Upstream Mediators for proteins found to be significant in the comparison of 96h Mitsui-7 (M-7) ALI versus submerged exposure. Sample sets were uploaded into IPA containing: protein ID, *p*-value (calculated from t-test), and $\log_2$ fold change (M-7 ALI / M-7 submerged) exposure. These heat maps demonstrate a greater amount of significant proteins found in the apical wash collection at the ALI compared to the apical media collection under submerged conditions. Note: *Z*-scores are considered significantly enriched when values are greater than 1.96 or less than -1.96 (α = 0.05).

TARGETED PROTEIN ASSAY

A

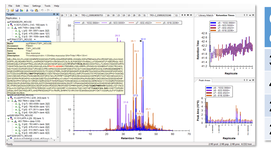
Pathways	Function	Target Proteins
NRF2-mediated oxidative stress response	Oxidative stress response	Heme Oxygenase-1, SOD 1 and 2, Thioredoxin
Acute phase response signaling	Inflammatory response	Complement Factor 3,4, Fibrinogen
Actin cytoskeleton signaling	Actin polymerization / stress fiber formation	Ezrin, F-actin, Integrin

B



C

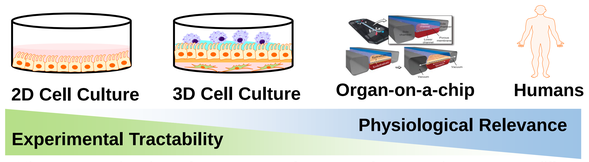
Criteria 1 (LC-MS/MS)
Unique to protein
Easily ionized by ESI
Reproducible chromatographic profiles
Free of easily oxidized or deaminated AA residues



Criteria 2 (stability check)
30 days at -20˚C
4 hours at 20 – 24 ˚C
24 hours at 4-8 °C
2 freeze/thaw cycles

Figure 7: (A) Candidate proteins used for targeted protein assay. **(B)** Schematic of targeted MS. **(C)** Criteria used to refine proteins suited for targeted MS assay development.

FUTURE DIRECTIONS



- Mine proteomic data for common features of expression across different experimental models following nanomaterial exposure.
- Identify novel mediators of stress response found in proteomic data not reported in single endpoint studies.
- Use global proteomic data to develop a multiplexed targeted protein assay to be used in place of antibody-based testing.
- Develop IATAs to assess pulmonary response to CNTs that includes existing data, exposure information, physico-chemical characterization, read-across, non-testing approaches (e.g., QSARs), omics data, and *in vitro* testing.
- Work is ongoing to develop an *in vitro* co-culture system to predict the development of pulmonary fibrosis after exposure to CNTs.[7,8]

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ACKNOWLEDGEMENTS

MSB acknowledges NC State University for Startup funds and the Center for Human Health and the Environment (CHHE) for supporting this work. GMH acknowledges the Toxicology Training grant award: T32 ES007046. Additionally, authors acknowledge support from the NIEHS: R01ES020897, RC2ES018772, and P30ES025128. GH, HB, and BRR acknowledge funding from the PETA International Science Consortium Ltd. and the Adolphe Merkle Foundaiton. MWCNTs (Mitsui-7, Mitsui & Co, Japan) were kindly provided by Prof. Vicki Stone, Heriot-Watt University, Edinburgh, UK,

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