DEVELOPMENT OF AN *IN VITRO* SYSTEM TO ASSESS THE INHALATION TOXICITY OF NANOMATERIALS

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INTRODUCTION

The increasing use of multi-walled carbon nanotubes (MWCNTs) in consumer products and their potential to induce adverse pulmonary effects following inhalation has led to much interest in better understanding the hazard associated with these nanomaterials (NMs). While the current regulatory requirement for substances of concern is a 90-day rodent inhalation test, the monetary, ethical, and scientific concerns associated with this assay led a group of international experts to convene in February 2015 to discuss approaches to evaluate the inhalation toxicity of MWCNTs. Pulmonary fibrosis was identified as a key adverse outcome linked to MWCNT exposure, and recommendations were made on the design of an *in vitro* assay that is predictive of the fibrotic potential of MWCNTs. Presented here are the preliminary data from the studies currently being conducted to develop such a model.

PHASE 1 (Completed)
- Workshop to develop recommendations on the design of *in vitro* system

PHASE 2 (In progress)
- Development of the system in one laboratory by testing MWCNTs and + / - controls

PHASE 3
- Testing in additional labs
- Testing of other materials

For more information, see: www.piscltd.org.uk/nanoworkshop/

PHASE 1: WORKSHOP TO DEVELOP RECOMMENDATIONS ON THE DESIGN OF AN *IN VITRO* SYSTEM

CHARACTERIZATION CONSIDERATIONS

Characterization stages:

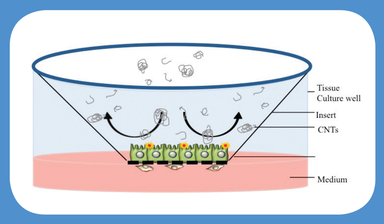
1. Pristine form
2. As aerosolized
3. Deposited dose
4. Post exposure

Characterization should include analysis of:
- Agglomerate structure (1, 2, 3, 4)
- (De)agglomeration (2, 3, 4)
- Impurity profile / content (1)
- Effective density (2)
- Bivariate length and diameter distribution (BVD) (1, 2, 3)
- Surface charge (1, 2)
- Surface area (1)
- Rigidity (1)
- Dustiness (1)
- Cellular uptake (4)

* The numbers (1, 2, 3,4) correspond to the characterization stages

Characterization of MWCNTs will be conducted at multiple stages

CONCEPTUAL MODEL



Mono- and co-culture systems including alveolar epithelial cells, macrophages, and fibroblasts will be exposed to MWCNTs at the air-liquid interface and under submerged conditions

DOSIMETRY

- Assessment of existing data to determine realistic exposure concentrations
- Consideration of mass, surface area, and number based dose metrics
- Use of *in silico* modeling (e.g., MPPD and ISDD models) to determine the deposited dose

RELEVANT ENDPOINTS

- Choice of biomarkers relevant to pulmonary fibrosis
- Application of adverse outcome pathways

PHASE 1 OUTPUTS

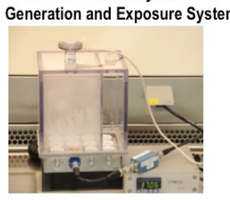
- Sharma M et al. (2016). Predicting pulmonary fibrosis in humans after exposure to multi-walled carbon nanotubes (MWCNTs). Arch Toxicol, In press.
- Clippinger AJ et al. (2016). Expert consensus on an *in vitro* approach to assess pulmonary fibrogenic potential of aerosolized nanomaterials. Arch Toxicol. doi: 10.1007/s00204-016-1717-8.
- Polk W et al. (2016). Aerosol generation and characterization of multi-walled carbon nanotubes exposed to cells cultured at the air-liquid interface. Part Fibre Toxicol, 13(1):20.

PHASE 2: DEVELOPMENT OF AN *IN VITRO* SYSTEM THAT IS PREDICTIVE OF PULMONARY FIBROSIS

TEST MATERIAL CHARACTERIZATION AND EXPOSURE

Two types of MWCNTs will be tested: Nanocyl 7000s (JRC NM 400) and Mitsui-7s.

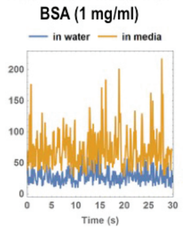
Vitrocell® Cloud system: NM Generation and Exposure System



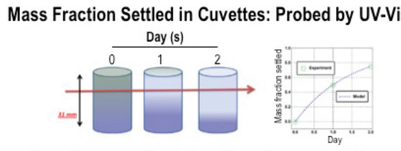
NM Deposition Assessed by QCM (µg/cm²)

MWCNT (Mitsui-7) concentration (µg/ml)	n=1	n=2	n=3	Mean	SD
5	0.34	0.64	-	0.49	0.21
25	1.22	1.32	1.43	1.32	0.11
50	1.82	1.58	1.39	1.6	0.22

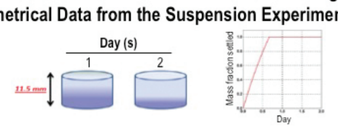
DLS Analysis of MWCNTs in Water and in Media with BSA (1 mg/ml)



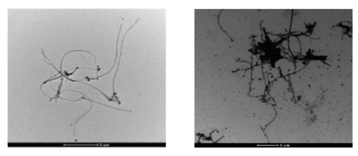
Mass Fraction Settled in Cuvettes: Probed by UV-Vis



Mass Fraction Settled *In Vitro*: Assessed Using Geometrical Data from the Suspension Experiments



MWCNT Deposition Assessed by TEM



ASSESSMENT OF EXISTING INFORMATION

MWCNTs Tested	*In Vitro*	*In Vivo*/Exposure Duration	Dose	Ref.
NM400, crushed NM400c, NM402, and MWCNTg 2400	Mouse lung (MLg), mouse embryonic fibroblasts (BALB-3T3), and human fetal lung fibroblasts (HFL-1)	C57BL/6 mice exposed to NMs via pharyngeal aspiration and fibrosis assessed after 60 days	*In vivo*: 12.5 - 100 µg *In vitro*: 7.5 - 30 µg/cm²	1
Nanocyl 7000	-	Male and female Wistar rats exposed head-nose to NMs for 6 hours/day for 13 weeks	0.1, 0.5, and 2 mg/m³	2
Nanocyl 7000	-	Male and female Wistar rats exposed to NMs nose-only for 6 hours/day, 5 days/week for 90 days	0, 0.1, 0.5, and 2.5 mg/m³	3
MWCNT1 (MWNT-7) and MWCNT2 (JRC)	RAW 264.7 cells	C57Bl6/J mice exposed to NMs via pharyngeal aspiration and fibrosis assessed after 8 weeks	*In vivo*: 1 time exposure to 1 mg/ml suspension/20 g bodyweight *In vitro*: 0.625, 2.5, and 10 µg/cm²	4

ABBREVIATIONS

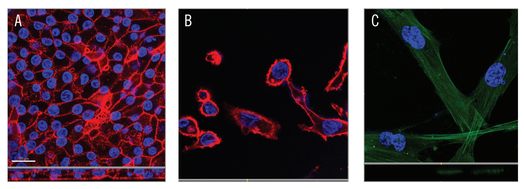
ALI	Air-Liquid Interface
BSA	Bovine Serum Albumin
DLS	Dynamic Light Scattering
ECM	Extracellular Matrix
GSH	Glutathione
IL	Interleukin
ISDD	*In Vitro* Sedimentation, Diffusion, and Dosimetry
JRC	Joint Research Centre
LSM	Laser Scanning Microscope
MPPD	Multiple-Path Particle Dosimetry
MWCNT	Multi-Walled Carbon Nanotube
NM	Nanomaterial
QCM	Quartz Crystal Microbalance
SEM	Scanning Electron Microscope
TBHP	tert-Butyl hydroperoxide
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumor Necrosis Factor Alpha
UV-Vis	Ultraviolet–Visible

REFERENCES

1. Vietti, G et al. (2013). Towards predicting the lung fibrogenic activity of nanomaterials: experimental validation of an in vitro fibroblast proliferation assay. Part Fibre Toxicol, 10:52.
2. Ma-Hock, L et al. (2009). Inhalation toxicity of multiwall carbon nanotubes in rats exposed for 3 months. Toxicol Sci, 112(2):468-481.
3. BASF Corporation (2011). Repeated dose; carbon nanotubes. CAS No 7782-42-5; ID No 8EHQ- 0411-17208C.
4. van Berlo, D et al. (2014). Apoptotic, inflammatory, and fibrogenic effects of two different types of multi-walled carbon nanotubes in mouse lung. Arch Toxicol, 88(9):1725-1737.

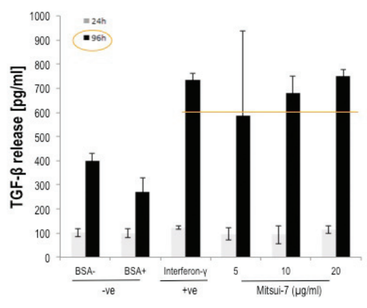
CELL SYSTEMS

Mono-Cultures



LSM images of suspension mono-cultures of (A) epithelial (A549), (B) macrophage (THP-1), and (C) fibroblast (MRC-5) cell lines. Cell morphology was assessed using immunostaining. Blue represents the nuclei and green represents the α-smooth muscle actin (MRC-5 cells) and red represents F-actin (A549).

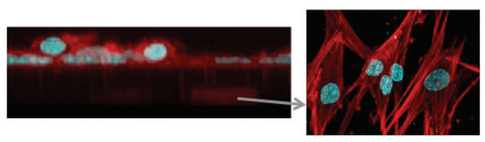
Observation(s):
A notable increase in TGF-β was observed in MRC-5 cells exposed to Mitsui-7 at all tested concentrations as compared to the negative controls at 96 hours.



Fibroblasts (MRC-5) were cultured on BD Falcon™ cell culture inserts (12 well) at a concentration of 0.5 x 10^6 cells/insert and were exposed in suspension to MWCNTs (Mitsui-7s dispersed in water with 0.1% BSA) at 3 concentrations (5, 10, and 20 µg/ml). After exposure for 24 or 96 hours, the supernatant was collected from the apical and the basolateral side of the insert. TGF-β levels were assessed relative to negative controls, i.e., cells were cultured in media with BSA (BSA+) and without (BSA-).The graph represents n=3 and the error bars represent the SEM.

Co-Culture

LSM images of a triple cell co-culture model of macrophages (THP-1), epithelial (A549), and fibroblast (MRC-5) cell lines. Cell morphology was assessed using immunostaining. Blue represents the nuclei and red represents the F-actin.



EpiAlveolar™ Model (MatTek Corporation, Ashland, Massachusetts)

LSM images of co-culture reconstructed with primary human epithelial and endothelial cells. Cell morphology was assessed using immunostaining. Blue represents the nuclei, red represents the F-actin, and green represents vascular endothelial-cadherin.