AN INTEGRATED APPROACH TO ASSESSING THE INHALATION TOXICITY OF NANOMATERIALS

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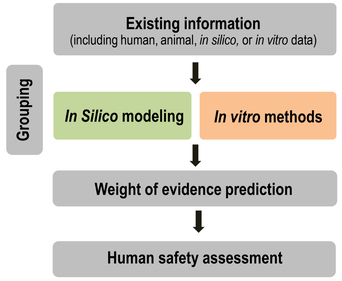
INTRODUCTION

Inhalation is the most prominent means of exposure to manufactured nanomaterials (NMs). While the current regulatory requirement for substances of concern in many jurisdictions is a 90-day rodent inhalation study, there are monetary, ethical, and scientific concerns associated with this test. Therefore, non-animal approaches are being sought to assess the hazard associated with these NMs. One such approach, using mono- and co-cultures of relevant human lung cells, has been developed to assess the potential of multi-walled carbon nanotubes (MWCNTs) to cause pulmonary fibrosis, a critical adverse outcome linked to prolonged NM exposure. When combined with other *in vitro* and *in silico* methods in an integrated approach, this system could be used to predict pulmonary toxicity and to enable effective risk assessment of substances including MWCNTs.



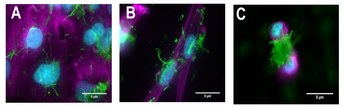
Integrated non-animal approach to assess the inhalation toxicity of aerosolized NMs (adapted from 1).

ASSESSMENT OF EXISTING INFORMATION

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

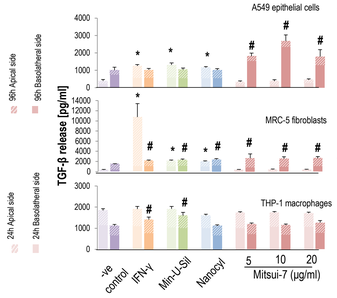
CELL SYSTEMS

Submerged Monolayer Cultures



Dark field images of (A) A549, (B) MRC-5, and (C) THP-1 cell lines exposed to Mitsui-7 MWCNTs suspension at 10 µg/mL after 24h. Green represents the MWCNTs, blue represents the nuclei, and magenta represents F-actin.

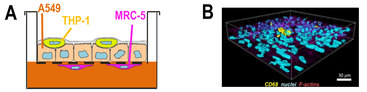
Observation
A notable increase in TGF-β release was observed following 96 (but not 24) hours of exposure of MRC-5 and A549 cells to 5, 10, or 20 µg/mL Mitsui-7 as compared to the negative controls



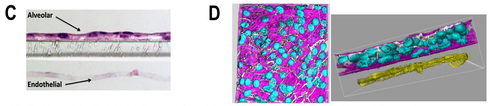
The three cell types were cultured and exposed in suspension to MWCNTs (Mitsui-7s and Nanocyl dispersed in water with 0.1% BSA) at different concentrations. After exposure for 24 or 96 h, the supernatant was collected and tested for different biomarkers of pro-inflammation and pro-fibrosis. Interferon-γ and Min-U-Sil were used as positive controls, and cells cultured in media with and without BSA were used as negative (-ve) controls. TNF-β release was assessed relative to negative controls. The graphs represent n=3; IFNγ=1µg/ml; error bars = standard error of mean; * = significant increase ($p < 0.05$) at 24h; and # = significant increase ($p < 0.05$) at 96h.

Three-Dimensional ALI Co-Cultures

Co-Cultures (Cell Lines)



EpiAlveolar™ Model (MatTek Corporation, MA)



Conceptual model (A) and LSM images (B and D) of a triple cell co-culture model of macrophages (THP-1), epithelial (A549), and fibroblast (MRC-5) cell lines (A and B) and EpiAlveolar™ model (D). A hematoxylin and eosin stained section of prototype EpiAlveolar™ model is also shown (C). Cell morphology was assessed using immunostaining in B and D. In B, yellow represents macrophage staining (CD68), blue represents the nuclei, and magenta represents the F-actin. In D, yellow represents vimentin, white represents E-cadherin, blue represents the nuclei, and magenta represents the actin bioskeleton.

The co-culture with cell lines and the EpiAlveolar™ models were exposed to MWCNTs (Mitsui-7s or Nanocyl dispersed in water with 0.1% BSA) at the ALI, at different concentrations. After 1 or 2 weeks of exposure, the supernatant was collected and tested for biomarkers of pro-inflammation and pro-fibrosis. Min-U-Sil was used as a positive control, and cultures in media with BSA were used as -ve controls. Levels of biomarkers were assessed relative to negative controls. Above are the representative graphs where TGF-β release was assessed. Error bars = standard error of mean, and the graphs represent n=4.

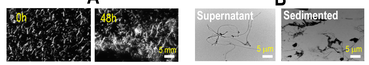
Observation
No significant increase in biomarkers of pro-inflammation and pro-fibrosis was observed in the co-culture or EpiAlveolar model after 1 or 2 weeks of exposure to different concentrations of MWCNTs or the positive control. Experiments are underway to test additional concentrations of MWCNTs and a different positive control, DQ12.

NM CHARACTERISATION, EXPOSURE, AND DOSIMETRY

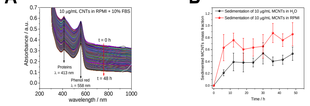
Two types of MWCNTs were tested: Nanocyl 7000s (JRC NM 400) and Mitsui-7s.

Suspension Exposure (Mono-Cultures)

MWCNT Deposition Assessed by (A) Enhanced Darkfield Microscope and (B) TEM



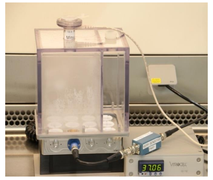
Kinetic Study of the (A) Sedimentation and (B) Mass Fraction Settled for Mitsui-7 in Cuvette Probed by UV-Vis Spectroscopy



Observation
A significant fraction of MWCNTs sediment after 24 hours but the kinetic rate depends on the suspension medium (water vs cell-culture media).

Air-Liquid Interface Exposures (Co-Cultures)

VITROCELL® Cloud system:
NM Generation and Exposure System

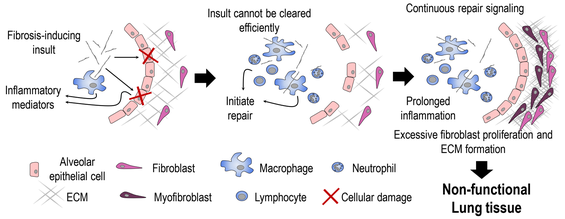


NM Deposition Assessed by QCM (µg/cm²)

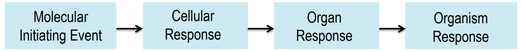
Suspension	Deposited Mass/Nebulization (µg/cm²)
BSA	0.2
Nanocyl	0.5
Min-U-Sil	0.7
Mitsui-7	1
Mitsui-7	2

IDENTIFICATION OF THE POTENTIAL MECHANISM OF ACTION

Mechanism of pulmonary fibrosis



Adverse outcome pathways



ABBREVIATIONS

ALI	Air-Liquid Interface
BSA	Bovine Serum Albumin
DLS	Dynamic Light Scattering
ECM	Extracellular Matrix
GSH	Glutathione
IL	Interleukin
IFN-γ	Interferon-gamma
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

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