Advanced *in vitro* liver and lung model development for engineered nanomaterial hazard assessment.

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Introduction:

Exposure to engineered nanomaterials (ENM) poses a potential risk to human and environmental health through long-term, repetitive, low-dose exposures. Current ENM hazard assessment tools are based on short term, high-dose exposures using simple 2D *in vitro* test systems, which lack environmental realism in terms of dose delivery, exposure duration and biological complexity. Thus, there is an urgent need for more realistic and predictive *in vitro* test systems for ENM safety assessment; **Physiologically Anchored Tools for Realistic nanOmaterial hazard aSsessment (PATROLS)** seeks to overcome these disadvantages.

PATROLS Aims to:

Establish and standardise a battery of innovative, next generation **hazard assessment** tools that **more accurately predict** adverse effects caused by **long-term (chronic), low dose** ENM exposure (example Figure 1) in human and environmental systems to **support regulatory risk decision making** and help **reduce** the need for **animal testing**.

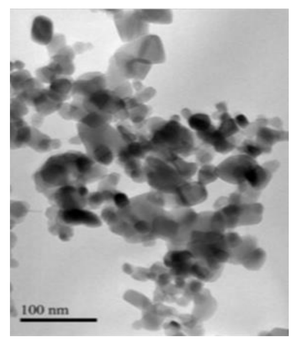
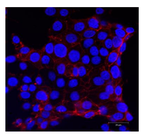


Figure 1. TEM image of Titanium Oxide (TiO_2).These specific EMNs are used for exposures to both models.
ENMs are from the European Commission's Joint Research Centre (JRC) and are standard particles used throughout the literature.
https://ec.europa.eu/jrc/en

Methods

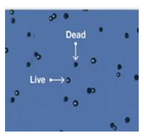
Characterisation will be completed during the growth and optimisation of the tissue models and after the addition of particles to the systems.

Cellular Structure and Growth:
- Histology (nuclear, tight junction & cytoskeleton morphology)
- Immunofluorescence Staining.



LSM X63 fluorescence image of normal cellular morphology of the A549 cell line.

Cytotoxicity Analysis:
- Cell counts
- Viability count (Trypan Blue & Propidium Iodide/Hoescht)



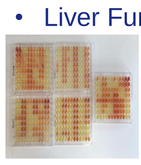
Trypan blue count – dead cells are those that are stained blue, while the live ones remain white (unstained).

http://www.genbiotech.net/oc/index.php?route=product/product&product_id=3937

Membrane Analysis:
- Transepithelial electrical resistance (TEER)
- Dextran Blue

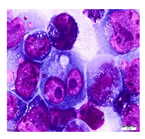
TEER measures the resistance over the membrane and therefore the membrane integrity.

Colourmetric Analysis:
- ELISAs
- LDH/Lactate Assays
- Liver Function Assays



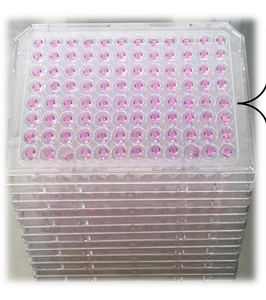
Colourmetric analysis plates

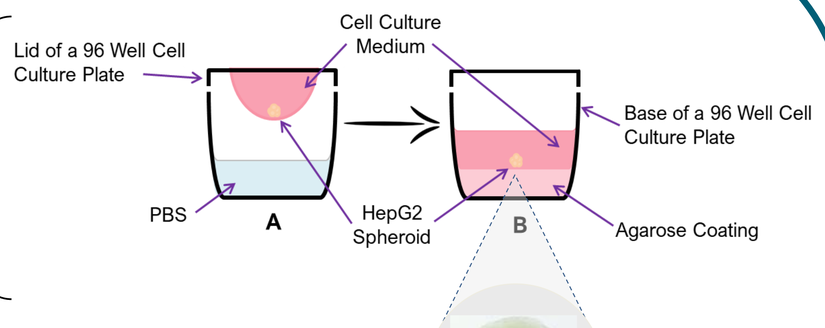
Genotoxicity Analysis:
- Cytokinesis Block Micronucleus (CBMN) Assay

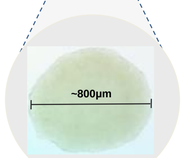


CellSens X63 image displaying binucleate formation with the presence of a micronucleus.

Liver Model







- 3D HepG2 liver spheroid models formed using a hanging drop set up over 3 days before transfer into 96-well plates with agarose coating.
- HepG2 cells seeded at 4000 cells/spheroid and remain viable for long-term (>14-days) culture and thus is able to support extended and repeated ENM exposures.

Figure 2: Diagrammatic representation of the HepG2 3D liver spheroid 96-well plate set up with transfer from hanging drop onto agarose coated wells.

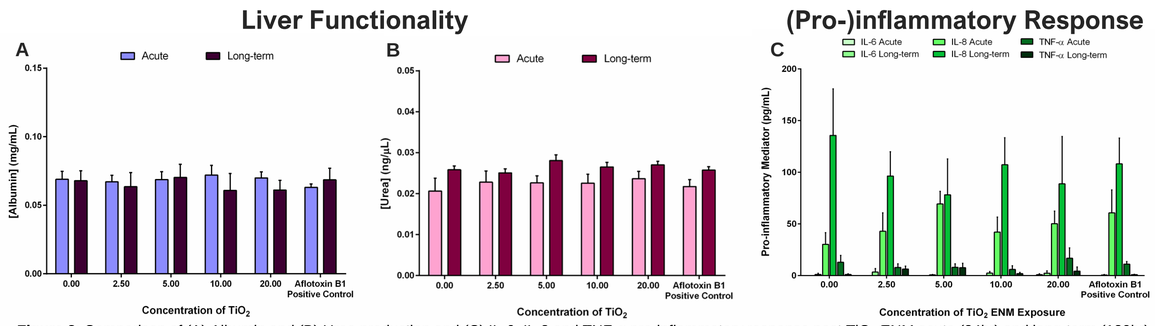


Figure 3: Comparison of (A) Albumin and (B) Urea production and (C) IL-6, IL-8 and TNF-α pro-inflammatory response post TiO_2 ENM acute (24hr) and long-term (120hr) exposures. Mean data (n=3) presented ± SEM.

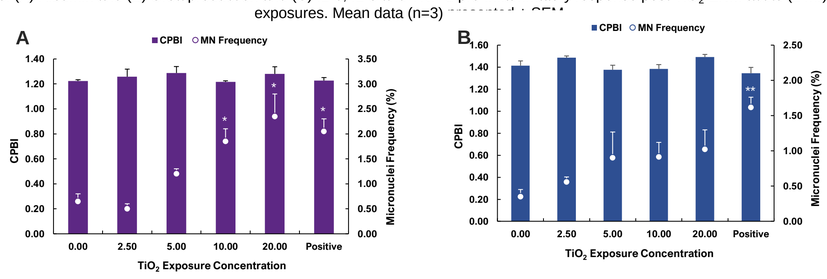
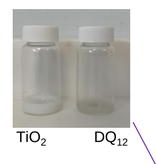


Figure 4: Cytotoxicity (CPBI) and genotoxicity (micronuclei frequency) assessment using the CBMN assay post (A) acute (24hr) and (B) long-term (120hr) exposure to TiO_2 ENM. Mean data (n=3) presented ± SEM. Significance indicated within a cell type: * = $p \leq 0.05$ & ** = $p \leq 0.01$.

Alveolar Model

- Composed of epithelial cells (A549 (ATCC® CCL-185)
- Grown on a transwell insert to initiate an air-liquid interface (ALI)
- Determined optimal time to switch to ALI was day 4 after seeding
- Determined optimal time to expose was 24 hours post-ALI switch



ENM sonicated for 16mins using a probe sonicator (The NANOGENOTOX dispersion protocol)

100µl of EMN Suspension

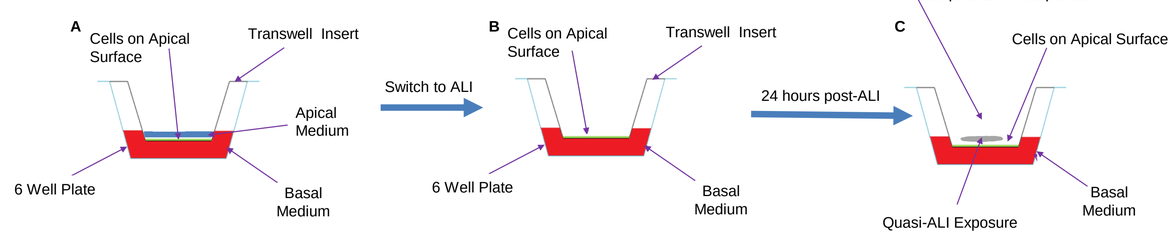


Figure 5: Diagrammatic representation of the growth (A), switch to an air-liquid interface (B) and exposure to ENM (C) of A549 cells.

Exposure to ENM:

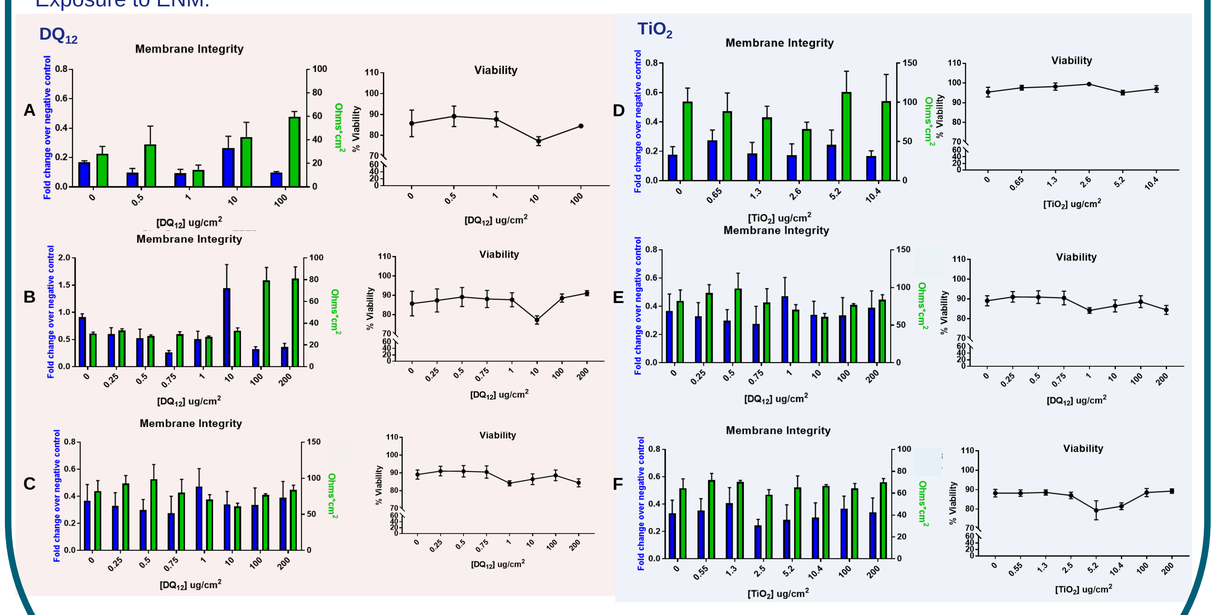
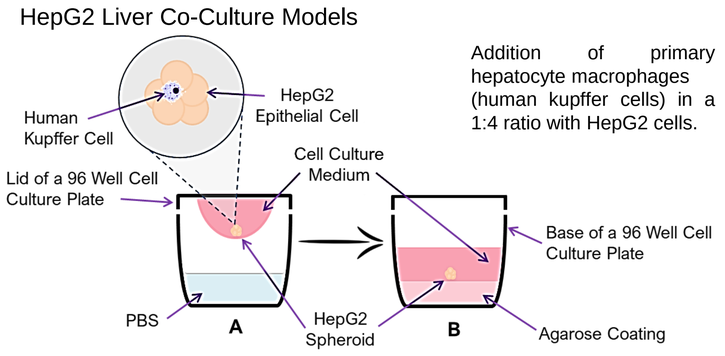


Figure 6: Membrane integrity and cellular viability after a quasi-ALI exposure to DQ_{12} and TiO_2 24 hours post-exposure (A and D), 48 hours post-exposure (B and E) and 72 hours post-exposure (C and F). N=3 subjects with all assays performed in triplicate. The data are presented as the mean ±SEM.

Further development of the models

Specific organ dynamics need to be taken into consideration, including movement (*i.e.* breathing motion) and flow dynamics (*i.e.* blood-flow) *via* the use of 3D printing and cell types (such as immune cells) found in the *in vivo* system.

HepG2 Liver Co-Culture Models



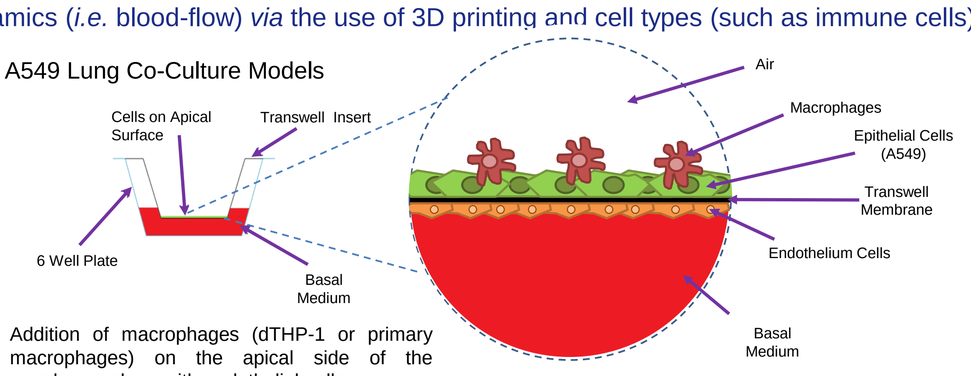
(Samantha Llewellyn 2019)

Addition of primary hepatocyte macrophages (human kupffer cells) in a 1:4 ratio with HepG2 cells.

Potential use of Quasi Vivo 600 from Kirkstall Ltd. Or the development and 3D printing of a chamber capable of regulating temperature, flow and mechanical movement.

http://www.kirkstall.com/brain/

A549 Lung Co-Culture Models



Addition of macrophages (dTHP-1 or primary macrophages) on the apical side of the membrane along with endothelial cells grown on the basal side of the membrane.

Conclusion:

Neither ENM, nor exposure scenario influenced the endpoints analysed. It is intended that following the successful development of such models, they can be used to establish advanced *in vitro* testing methods that will contribute towards the reduction of *in vivo* testing approaches across toxicology and drug discovery research

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Special thanks to Swansea University IVTG group and all WP3 & WP4 members of PATROLS as well as the entire consortium.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

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