Advancing liver and lung in vitro models to realistically assess human health hazards of engineered nanomaterials.

Kirsty Meldrum, Samantha Llewellyn, Stephen J. Evans, Ume-Kulsoom Shah, Joana Amaral Duarte De Moura, Gareth J. S. Jenkins, Martin J. D. Clift and Shareen H. Doak

In Vitro Toxicology Group, Swansea University Medical School, Swansea University, Singleton Campus, Centre for NanoHealth, Institute of Life Sciences, SA2 8PP, Swansea, Wales, UK

Introduction:

Due to the constant increase in their production, exposure to engineered nanomaterials (ENM) poses an inevitable health risk to both humans and the environment through long-term, repetitive, low-dose exposures. The majority of literature focuses on short-term, high-dose exposures. Systems are being developed to allow key long-term studies to be achieved *in vitro.* Current *in vitro* models have both advantages and disadvantages (Table 1.), this project aims to develop these models to create a model that overcomes these disadvantages. This will be facilitated with the use of standard particles that can be bought and used as a comparison to results already in the literature.

Physiologically Anchored Tools for Realistic nanOmaterial hazard aSsessment (PATROLS) is an EU Horizon2020 funded research and innovation project.

Table 1. Current *in vitro* models advantages and disadvantages. Not one model is perfect, therefore work needs to be done to optimise the models we currently have.

	Heterogeous Cell Population	3D Conformation	Chemical Cues	Mechanical Stimulus	Low Cost	Easy use	Low Equipment/ Facilities
2D plastic cell culture	✓	✗	✗	✗	✓	✓	✓
Inserts	✓	✗	✗	✗	✓	✓	✓
Organoids	✓	✓	✓	✗	✓	✓	✓
Microfluids	✗	✗	✗	✓	✗	✗	✗
Synthetic Scaffolds	✗	✓	✓	✗	✓	✓	✓
Biological Scaffolds	✗	✓	✓	✗	✓	✓	✓
3D Bioprinting	✗	✓	✓	✓	✗	✗	✗

Current *in vitro* models advantages (✓) and disavantages (✗).

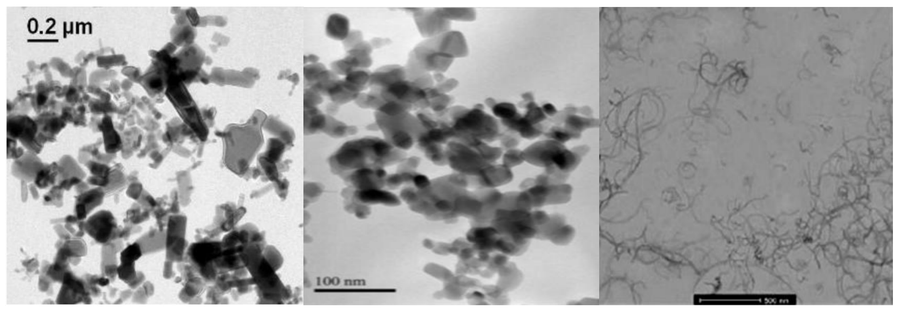


Figure 1. TEM images from Zinc Oxide (ZnO), Titanium Oxide (TiO_2) and a Multi-Walled Carbon Nanotube (MWCNT). All ENMs are from the European Commission's Joint Research Centre (JRC) and are standard particles used throughout the literature.
https://ec.europa.eu/jrc/en

Aims:

Establish, characterise and implement a plethora of innovative, physiologically realistic 3D in vitro models that can be applied as dynamic tools in deducing the potential ENM hazard posed to humans and the environment.

Methods:

Advancing 3D liver and lung co-cultures models using both primary cells and cell lines, fully characterise these models and then exposing these to standard ENMs that are commercially available (Figure 1.).

Liver Model

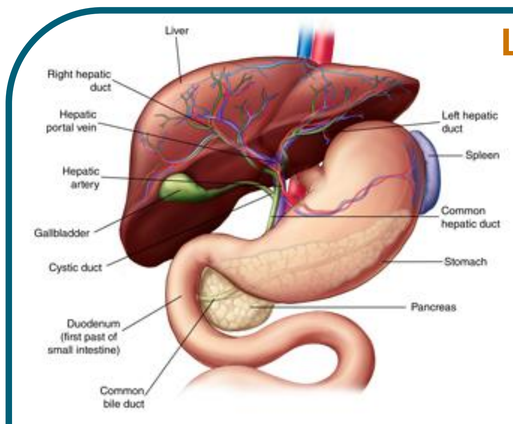
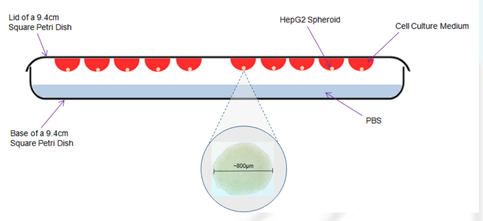


Diagram of the Liver
https://www.saintlukeskc.org/health-library/how-liver-works



Example of hanging drop set up. HepG2 and HepaRG cells in spheroid formations (around 0.8mm in diameter)

- 3D HepG2 mono- and co-culture models are produced with the hanging drop method
- HepG2 cells are seeded at a density of 5000 cells/mL and left to form naturally.

Once formed, the spherical 3D structure mimics the complex extracellular matrix architecture and intricate cell-cell interactions, aspects vital when representing *in vivo* environments.

Lung Model

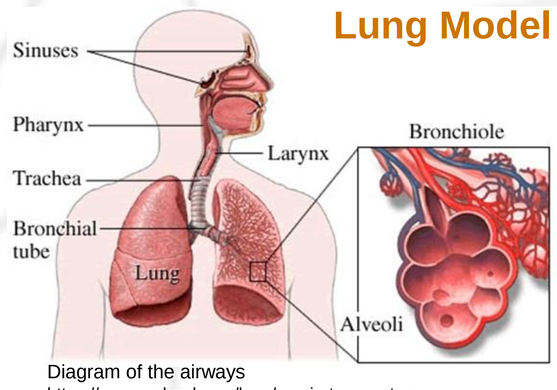
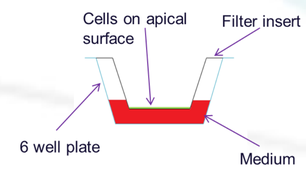


Diagram of the airways
https://www.webmd.com/lung/respiratory-system



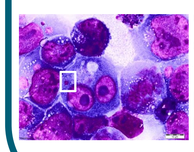
Example of ALI set up, cells at the apical side exposed to air and medium on the basal side

- Composed of epithelial cells (either A549 (cell line) or SAECs (primary cells))
- Grown on a transwell insert to initiate an air-liquid interface (ALI)
- Once optimisation of both these cell types has been completed, one will be carried forward to be used in future experimental work.

Characterisation

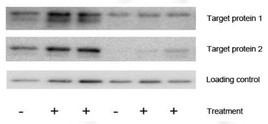
Characterisation will be completed during the growth and optimisation of the tissue models and after the addition of particles to the systems.

<u>Genotoxicity Analysis:</u>
- Cytokinesis Block Micronucleus Assay

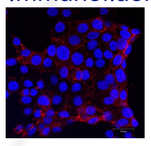


CellSens X63 image displaying binucleate formation with the presence of a micronucleus.

<u>Protein Analysis:</u>
- Western Blots
- rtPCR (*CYP450 Activity*)

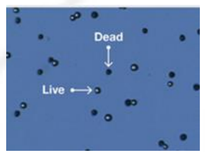


<u>Cellular Structure and Growth:</u>
- Histology (nuclear, tight junction and cytoskeleton morphology)
- Immunofluorescence Staining.



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

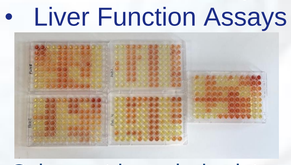
<u>Cytotoxicity Analysis</u>:
- Cell counts
- Viability count (*Trypan Blue & Propidium Iodide/Hoescht*)



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

<u>Colourmetric Analysis</u>:
- ELISAs
- LDH/Lactate Assays
- Liver Function Assays



Colourmetric analysis plates

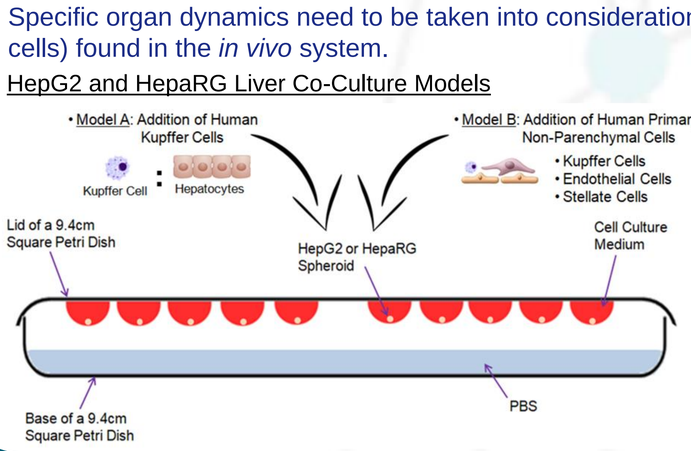
<u>Membrane Analysis</u>:
- Transepithelial electrical resistance (TEER)
- Dextran Blue

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

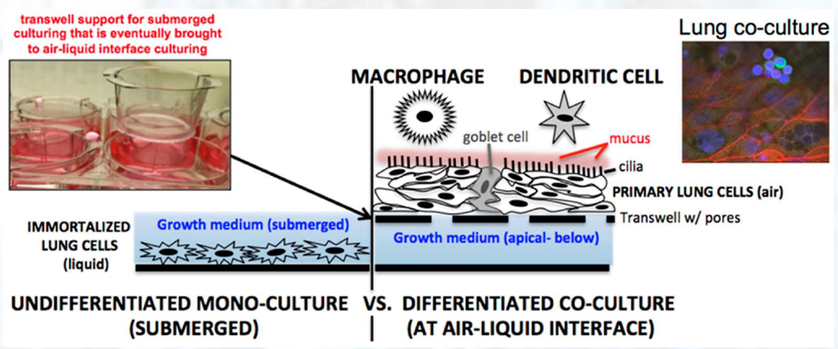
Further development of the models

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.

<u>HepG2 and HepaRG Liver Co-Culture Models</u>



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/



Addition of immune cells to the lung epithelial cells in order to create a co-culture which attempts to represent the *in vivo* system.
Powell, J.D.; Straub, T.M. Advances and Remaining Challenges in the Study of Influenza and Anthrax Infection in Lung Cell Culture. *Challenges* **2018**, *9*, 2

Conclusion:

It is intended that following the successful development of such models, they can be used to establish advanced testing methods that will contribute towards the reduction of *in vivo* testing approaches across toxicology and drug discovery research

References:
- https://www.patrols-h2020.eu
- https://ec.europa.eu/jrc/en
- https://www.saintlukeskc.org/health-library/how-liver-works
- https://www.webmd.com/lung/respiratory-system
- http://www.genbiotech.net/oc/index.php?route=product/product&product_id=3937
- Powell, J.D.; Straub, T.M. Advances and Remaining Challenges in the Study of Influenza and Anthrax Infection in Lung Cell Culture. *Challenges* **2018**, *9*, 2
- http://www.kirkstall.com/respiratory-models-using-quasi-vivo/

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

@PATROLS_H2020 https://www.patrols-h2020.eu