Advanced Engineered Nanomaterial (ENM) Exposure Regimes for more Realistic Dosing Scenarios on 3D Liver Models *In Vitro*.

S.V. Llewellyn[1], G.E. Conway[1], A.A. Kämpfer[2], J.G. Keller[3], K.Vilsmeier[3], D. Ag Seleci[3], R.P.F. Schins[2], G.J.S. Jenkins[1], M.J.D. Clift[1], W.Wohlleben[3] and S.H. Doak[1]

[1]*In Vitro* Toxicology Group, Institute of Life Sciences 1, Swansea University Medical School, Singleton Campus, Swansea, UK, SA2 8PP
[2]Leibniz Research Institute for Environmental Medicine, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany
[3]Advanced Materials Research, Dept. of Material Physics and Analytics and Dept. of Experimental Toxicology and Ecology, BASF SE, 67056 Ludwigshafen, Germany
***S.V.Llewellyn@swansea.ac.uk**

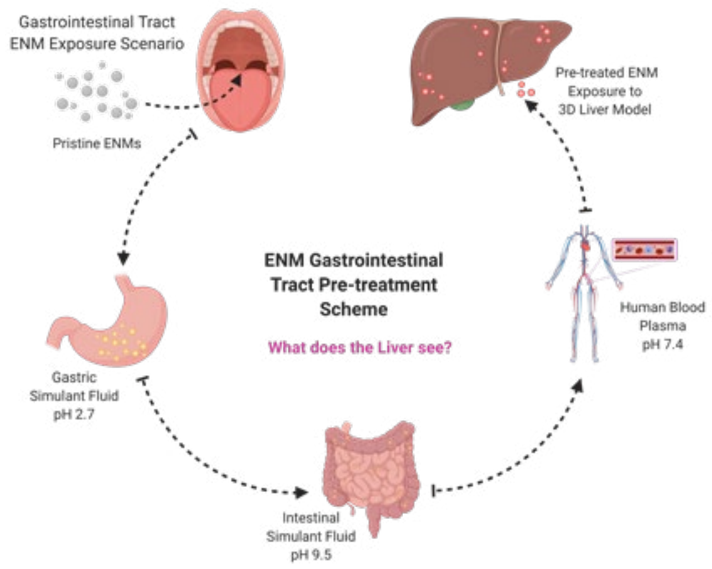

Figure 1: Schematic diagram displaying the ENM pre-treatment protocol to emulate ENM exposure on the liver *via* ingestion.

Introduction:

Organ systems such as the liver, are secondary sites of exposure for engineered nanomaterials (ENMs), following systemic translocation from primary exposure sites as a result of injection, ingestion, inhalation or dermal exposure. Hence, it is highly unlikely that the liver would be exposed to "pristine" ENM as interaction with a variety of molecules in varying cellular compartments (Fig. 1) can transform the physico-chemical properties (*e.g*. surface chemistry, protein corona, morphology and dissolution) of the ENM.

Aim:

The objective of this study was to: 1) evaluate the toxicological response of repeated, low-dose ENM exposure regimes and 2) determine the impact of pre-treating ENMs with physiologically relevant simulant fluids prior to exposure, using 3D hepatic cultures.

Methods:

In vitro 3D hepatocyte models were developed using an immortalised cell line (HepG2), which are viable for long-term culture (>14 days) and able to support both long-term and repeated ENM exposures (Fig. 2). Their ability to quantify a range of toxicological endpoints (*e.g.* liver function, (pro-)inflammatory response, cytotoxicity and genotoxicity) has been evaluated using a range of JRC reference ENMs (*e.g.* TiO_2 and ZnO) (Fig. 3) across both short- (24hr) and long-term (120hr) low-dose exposure regimes.

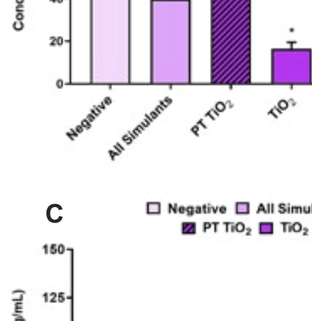

Figure 3. TEM images of Titanium Dioxide (TiO_2) ENMs from the European Commission's Joint Research Centre (JRC). https://ec.europa.eu/jrc/en

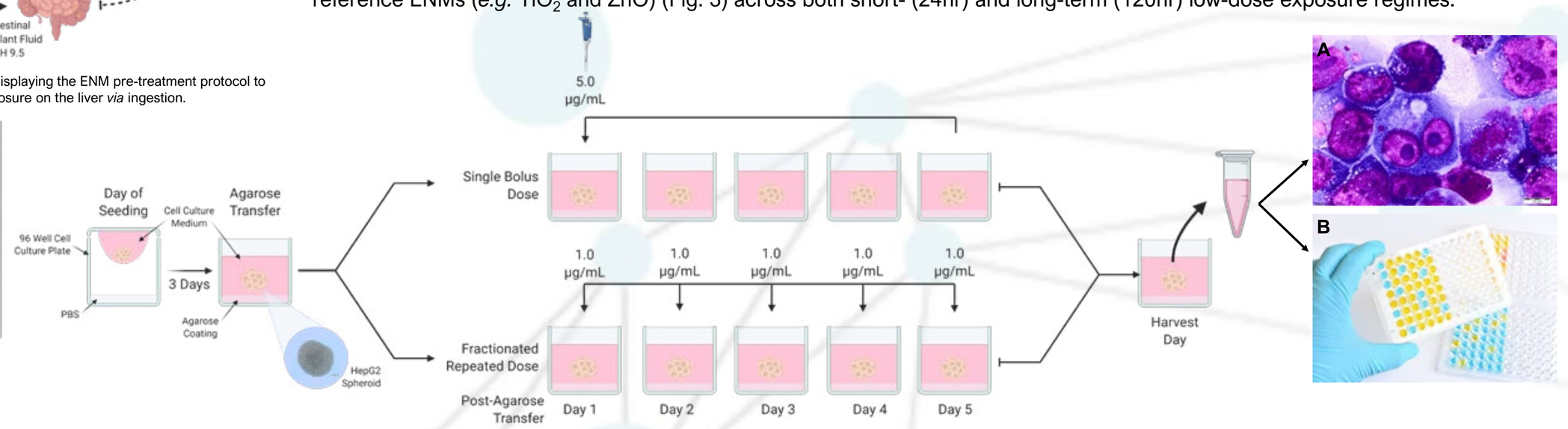

Figure 2: Schematic representation of the long-term (120 hour) single, bolus ENM exposure regime and the fractionated, repeated ENM exposure regime assessed using 3D HepG2 spheroids and key biochemical endpoint analysis techniques selected. (A) CellSens X63 image displaying binucleate formation following the cytokinesis-block micronucleus (CBMN) assay with the presence of a micronucleus. (B) (Pro)-inflammatory ELISAs for Interleukin 8 (IL-8), Interleukin-6 (IL-6) and Tumor Necrosis Factor Alpha (TNF-α). Created with Biorender.com

Results:

Pre-treatment of ENMs with GIT simulant fluids

(Pro-)Inflammatory Response

Figure 4: Comparison of IL-8 (A & B) and TNF-α (C & D) pro-inflammatory response post acute (24hr) and long-term (120hr) exposure to both pristine TiO_2 and Ag ENMs and TiO_2 and Ag ENMs pre-treated (PT) with GIT simulant fluids. Mean data of three biological replicates, analysed in triplicate (n=9) presented ± SD. Red dotted line represents the mean positive control response induced by 50 µg/mL of TNF-α protein (NBP2-35076-50 µg, Biotechne, UK). Significance indicated in relation to the negative control: * = $p \leq 0.05$ with significance between groups indicated as: φ = $p \leq 0.05$.

Genotoxicity Assessment

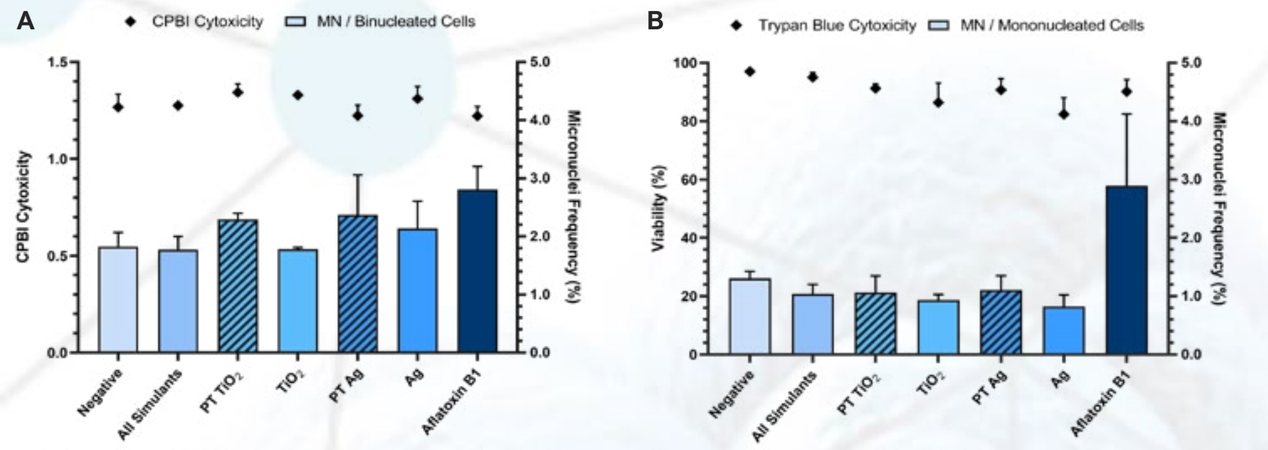

Figure 5: Cytotoxicity (CPBI / Trypan Blue Viability) and genotoxicity (micronuclei frequency) response in HepG2 spheroids following acute (24hr) and long-term (120hr) exposure to both pristine and pre-treated (PT) TiO_2 and Ag ENMs using the micronucleus (MN) assay. For acute exposures, 1000 binucleated cells were scored per dose per replicate using the cytokinesis-block version of the MN assay (3000 binucleate cells scored in total), whilst 2000 mononucleated cells per dose per replicate were scored using the mononuclear version of the assay (6000 mononucleated cells scored in total). Mean data of three biological replicates (n=3) presented ± SD.

- Both **PT TiO_2** and **PT Ag ENMs** were shown to **induce an elevated IL-8** and **TNF-α response** compared to their pristine counterparts; with PT TiO_2 and PT Ag inducing a significant increase in IL-8 (Fig. 4A) and TNF-α (Fig. 4C) respectively, following acute 24 hour exposure. Yet, following **long-term exposures**, this effect was **no longer observed** (Fig. 4B & D).
- **Pre-treating ENMs** did **not significantly impede liver functionality nor induce elevated cytotoxicity or genotoxicity** (Fig 5.) in 3D liver spheroids.
- **No significant difference** in **DNA damage response** was seen whether **a fractionated or bolus long-term ENM exposure regime was applied**, Fig. 6.

Repeated ENM Exposure Regimes

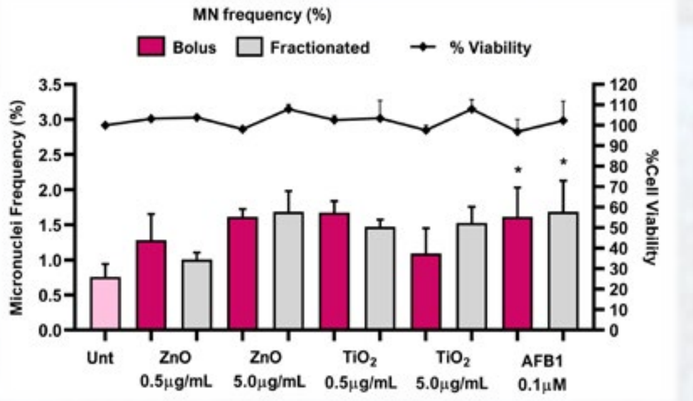

Figure 6: Cytotoxicity (CPBI) and genotoxicity (micronuclei frequency) assessment using the micronucleus assay post long-term (120hr) exposure to ZnO and TiO_2 ENM. Mean data (n=2) presented ± SD. Significance indicated in relation to the negative control: * = $p \leq 0.05$.

Conclusion:

- The impact of GIT simulated ENM transformation upon toxicological outcomes at a potential secondary organ of exposure (Liver) have been demonstrated using an advanced *in vitro* 3D culture test system.
- In addition, a physiologically relevant tiered testing strategy with which to assess the biological effects of ENM transformation that arise as the ENM traverse a cascade of biological compartments has been developed.
- Further work into independent toxicological effects (*e.g.* (pro-)inflammatory effects) of fractionated, repeated ENM dosing needs to be further evaluated.

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