Supplementary Information

Simulating Nanomaterial Transformation in Cascaded Biological Compartments to enhance the Physiological Relevance of *In Vitro* Dosing Regimes: Optional or Required?

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1. Simulant Pre-treatment Fluid Composition and ENM Characterisitics

Table S1: Media Composition of physiological relevant fluids, IUF_G, IUF_I, LSF, PSF, which were reproducibly standardised across all labs in the current contribution.

IUF_G (Gastric Solution) pH 2.7		[mg/L]
Sodium chloride	NaCl	1980
Hydrochloriric acid	HCl	to pH 2.7

IUF_I (Intestinal Solution) pH 9.5		[mg/L]
Sodium chloride	NaCl	2000
Sodium hydrogencarbonate	$NaHCO_3$	3580
Disodium carbonate	Na_2CO3	840

PSF (Stefaniak et al.) pH 4.5		[mg/L]
Sodium phosphate dibasic anhydrous	Na_2HPO_4	171
Sodium chloride	NaCl	6650
Sodium sulfate anhydrous	Na_2SO_4	71
Calcium chloride dehydrate	$CaCl_2x2H_2O$	29
Glycine	$C_2H_5NO_2$	450
Potassium hydrogen phthalate	$(1\text{-}(HO_2C)\text{-}2\text{-}CO_2K)\text{-}C_6H_4)$	4084.6
Alkylbenzyldimethylammonium chloride	(ABDC)	50

LSF (Modified Gamble (citrate)) pH7.4		[mg/L]
Sodium Chloride	NaCl	3208
Sodium Hydroxide	NaOH	1888
Citric Acid xH2O	Citric Acid xH2O	5424
Calcium chloride dehydrate	$CaCl_2x2H_2O$	29
Sodium phosphate dibasic anhydrous	Na_2HPO_4	171
Sodium sulfate anhydrous	Na_2SO_4	40
Magnesium Chloride	MgCl2x6H2O	106
Glycine	$C_2H_5NO_2$	59
Trisodium citrate	Na3citrate x2H2O	76
Sodium tartrate	Na2tartrate x2H2O	90
Sodium pyruvate	Na-pyruvate	86
90% lactic acid	$C_3H_6O_3$	79

Table S2: Physical-chemical properties of the ENMs evaluated.

Property & Method of Analysis	**Unit**	**Amorphous SiO_2**	**SiO_2 NM200**	**Ag Sigma (Cat#: 576832, PVP)**	**Ag NM300**
Composition: CAS	n/a	7631-86-9		7440-22-4	
XRD: composition, crystallinity	n/a	amorphous SiO_2	amorphous SiO_2	Ag	Ag_2O + Ag (93% : 7%)
XRF: impurities <0.1%, <1% exact value if >1%	%	<0.1% (CaO, CuO)	SO_3 2.62%, Na_2O 1.44%; <1% (Cl, Al2O3); <0.1% (CaO, TiO_2, Fe_2O_3, K_2O, CuO, NiO, ZrO_2, ZnO)	<1% (Pd, Cl); <0.1% (Rh, Fe, Cu, Ni)	<1% (CaO, P_2O_5, Pd); <0.1% (Cl, CdO, K_2O, Fe_2O_3, SiO_2, CuO, NiO, MoO_3)
TGA/DTG: weight loss	%	nd	-3.29	-3.37	- 83.63
TEM/SEM: constituent (primary) particle size and shape	D50, nm	8.3 ± 3	35.5	30.0 ± 23.9	7.2
	3D / 2D / 1D	3D; mean aspect ratio 1.30 ± 0.93; mean circularity 0.24 ± 0.06	Mean aspect ratio: 1.57; Mean circularity: 0.41	3D; mean aspect ratio 1.36 ±0.3; mean circularity 0.88 ± 0.09	3D; mean aspect ratio 1.20; mean circularity 0.98
Chemical Nature of the Surface: coatings	n/a	none	none	PVP functionalization	Identified two TGA peaks
XPS: chemical nature of the surface	atom%	Si 30.8%, O 69.2%	C 4.1%, O 70.8%, Si 24.1%, S 0.06%, Na 1.0%	Ag 38.9%, C 47.6%, O 13.5%	Ag 1.4%, C 71.3%, O 27.2%
BET: specific surface area	m^2/g	193	166	6.43	not measurable (suspension)
He pycnometer: density	g/cm^3	3.9	2.19	8.36	not measurable (suspension)
IEP: Surface charge	pH	3.5	3.3	< 3	3.9
Zeta Potential pH7: Surface charge	mV	-35	-22	-30	-22
Water contact angle: surface hydrophobicity	°	77.1 ± 1.4°	<10°	140.8 ± 1°	not measurable (suspension)

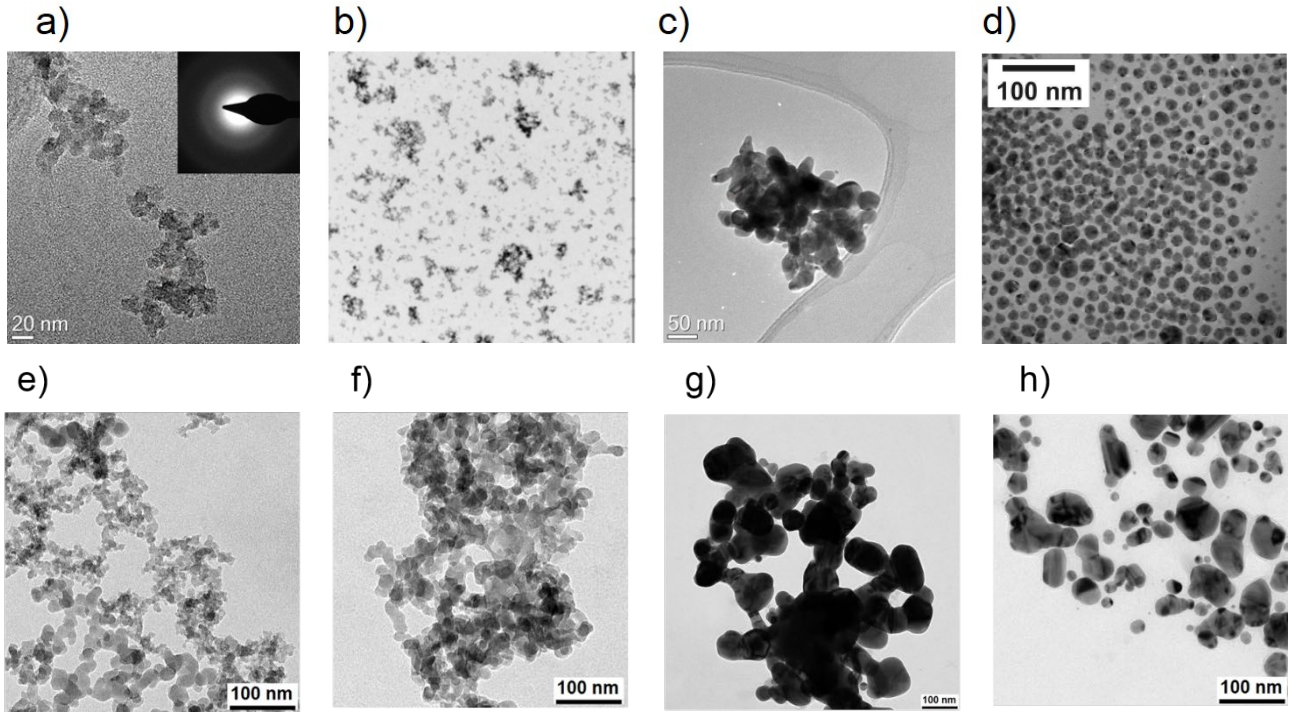

Figure S1: TEM micrographs of the ENM: a)-d) show the pristine ENM, and e)-h) show the aged ENM after sequential IUF_S and IUF_I treatment. a,e) Amorphous SiO_2; b,f) SiO_2 NM200; c,g) Ag Sigma; d,h) Ag NM300. Figure d) reproduced from Klein et al. (2011) JRC report 60709 (DOI 10.2788/23079).

Figure S2: XPS analysis of transformation induced by the IUF (=IUF_G + IUF_I) GIT pre-treatment of the two Ag materials. The results represent triplicate testing, and the small numbers report the atom-% composition of the surface. As controls, the materials are analyzed in the original state (no dispersion), and after dispersion in deionized water with sonication. The IUF GIT pre-treatment does not use sonication, but simulates a chemically aggressive compartment. The Ag NM300 is already present in oxidized form before the GIT pre-treatment, and is already initially covered by 73% C and 23% O from the polymer functionalization. With the additional remains from the GIT pre-treatment (observed as Na in both cases), the total Ag content on the surface remained below the detection limit of 0.5%, such that no oxidation state could be determined for Ag NM300 after GIT pre-treatment.

Table S3: Ion concentrations in mg/L of a total solid content of 4000 mg/L of Ag Sigma and amorphous SiO_2 after filtration through a 5kDa membrane in different media. Concentrations measured through ICP-MS.

		Ag Sigma	SiO_2 amorphous
Medium	FBS	0.021	4.43
	LSF	0.216	6.67
	PSF	0.000	1.53
	IUF_G	0.025	0.63
	IUF_I	0.032	8.48
	IUF (G to I)	0.034	4.58
	FBS to PSF	0.102	1.44
	IUF to FBS	0.344	1.34
	LSF to PSF	0.585	2.07
	LSF to FBS	0.392	10.73
	IUF to PSF	0.025	2.66

Table S4: Particle size distribution descriptors D10, D50 and D90 after single incubation for Ag Sigma and amorphous SiO_2 and SiO_2 NM200.

		D10 nm	D50 nm	D90 nm
Ag Sigma	FBS	14	63	156
	LSF	7	409	936
	PSF	382	545	666
	IUF_G	33	149	352
	IUF_I	26	88	289

		D10 nm	D50 nm	D90 nm
SiO_2 amorphous	FBS	117	263	598
	LSF	726	1611	2200
	PSF	141	569	1239
	IUF_G	43	63	96
	IUF_I	117	263	598

Table S5: Particle size distribution descriptors D10, D50 and D90 after sequential incubation for Ag Sigma, Ag NM300, amorphous SiO_2 and SiO_2 NM200. The code "FBS to PSF" means that the particles are first dispersed by stirring in FBS, incubated, then second medium PSF added, resuspended by sonication, incubated, then analyzed without further sample preparation directly in PSF.

		D10 nm	D50 nm	D90 nm
Ag NM300	IUF_G to _I	8	10	16
	FBS to PSF	10	12	19
	IUF to FBS	9	12	19
	LSF to PSF	10	12	19
	LSF to FBS	10	12	18
	IUF to PSF	10	12	18

		D10 nm	D50 nm	D90 nm
SiO_2 NM200	IUF_G to _I	499	1840	4393
	FBS to PSF	842	1991	2670
	IUF to FBS	181	1124	1810
	LSF to PSF	654	1480	2364
	LSF to FBS	682	1541	2069
	IUF to PSF	655	1566	2566

		D10 nm	D50 nm	D90 nm
Ag Sigma	IUF_G to _I	47	377	704
	FBS to PSF	6	36	115
	IUF to FBS	264	601	828
	LSF to PSF	327	603	854
	LSF to FBS	23	121	319
	IUF to PSF	264	622	952

		D10 nm	D50 nm	D90 nm
SiO_2 amorphous	IUF_G to _I	57	98	167
	FBS to PSF	308	2173	2774
	IUF to FBS	250	1248	1984
	LSF to PSF	434	1171	1686
	LSF to FBS	585	1691	2364
	IUF to PSF	486	1680	2344

2. Additional Control Experiment on Sequential Incubations

The effects observed by sequential incubation may be a trivial result of prolonged incubation. To check this hypothesis, we performed the sequential incubation protocol with one and the same medium. We selected the most labile (most reactive) material for this test, Ag Sigma. Table S6 assembles the results obtained. The comparison of sequential incubation vs. same-same incubation shows that it is not the length of incubation, and not a single medium, but indeed the sequence that is decisive for the final size of Ag Sigma:

- PSF to PSF is markedly different from FBS to PSF (numbers in brown).
- IUF to IUF is markedly different from IUF to FBS (numbers in red).
- LSF to LSF is markedly different from LSF to FBS (numbers in grey)

Table S6: Particle size median (D50) after normalization to median of the size distribution when dispersed directly into the second medium. The color code of the NUMBERS identified values that should be compared to each other. The color code of the CELLS is normalized on the entire data set to highlight cases where the sequential incubation leads to results very similar (white) or lower (blue) or higher (red) agglomeration as compared to single incubation in either the first or the second medium of the sequence.

		relative to 1st medium	relative to 2nd medium
Ag Sigma	FBS to PSF	57%	7%
	IUF to FBS	159%	954%
	LSF to PSF	147%	111%
	LSF to FBS	30%	192%
	IUF to PSF	165%	114%
	IUF to IUF		149%
	LSF to LSF		100%
	PSF to PSF		128%

3. Additional Results on Genotoxicity

The pre-treatment of Ag Sigma ENM with artificial GIT digestive fluids significantly reduced the cytotoxic potential in both confluent Caco-2 and E12 cells compared to the pristine particles. Interestingly, the pre-treatment of the particles did not consistently affect observed DNA damage in the cell lines. Whereas the exposure to both pristine and PT Ag Sigma caused a significant increase in DNA damage in Caco2- cells at 80µg cm^{-2}, this was only the case for pristine Ag Sigma ENM in E12 cells. Following exposure to PT Ag Sigma, the measured increase in tail intensity was not significant for concentrations up to 80µg cm^{-2} and significantly less DNA in tail was measured compared to the pristine concentration equivalent.

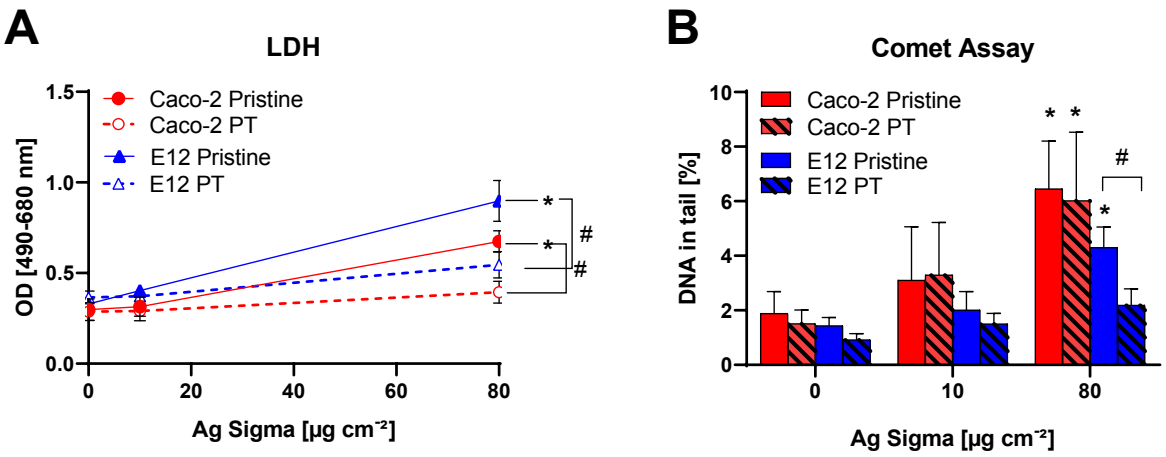

Figure S3: LDH release (A) and DNA damage (B) in Caco-2 (red) and E12 (blue) monocultures after 24hrs exposure to pristine or PT Ag Sigma ENM (Average ± SD, N=3; *$p \leq 0.05$ compared to respective control; #$p \leq 0.05$ compared to corresponding pristine Ag Sigma concentration by One-way ANOVA and Tukey's *post hoc* test)

Cytokinesis-block Micronucleus (CBMN) Assay for Genotoxicity

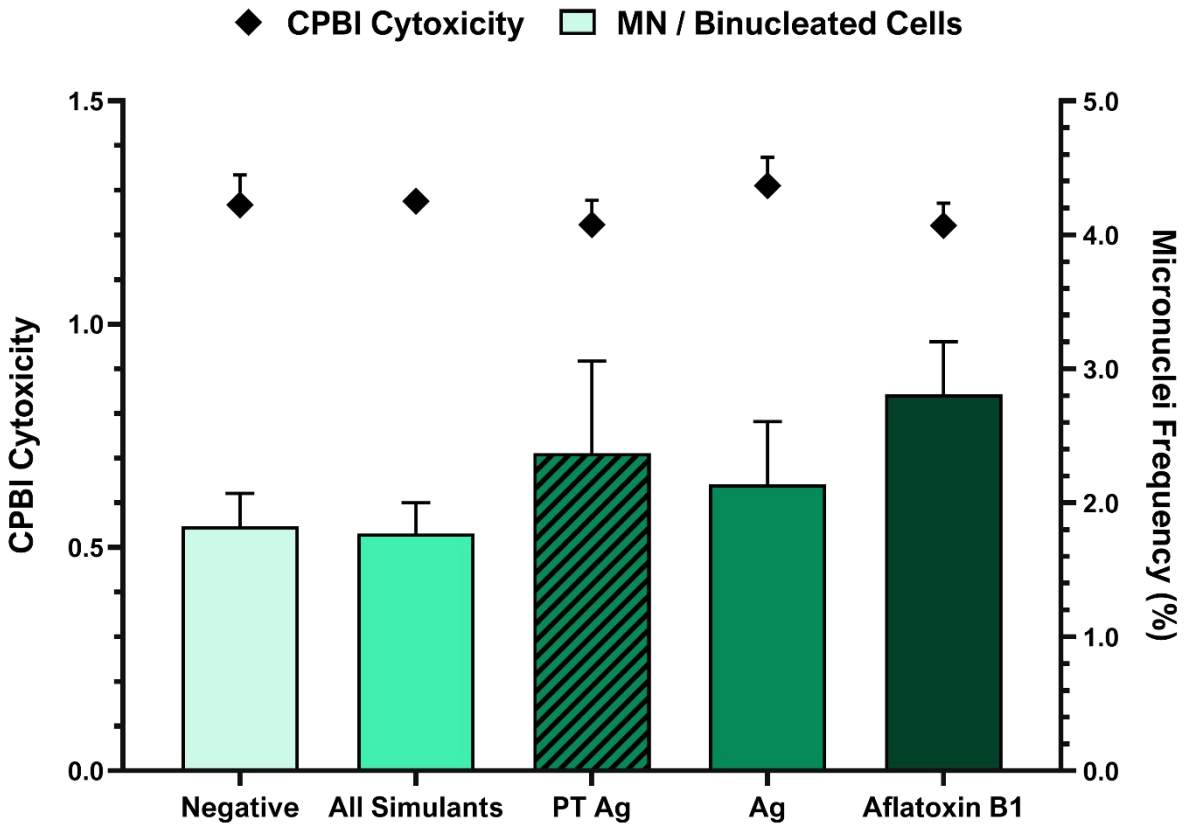

Figure S4: Genotoxicity and cytotoxicity response in HepG2 spheroids following acute 24 hour exposure to both pristine and PT Ag ENMs using the cytokinesis-block micronucleus (CBMN) assay with the cytokinesis-block proliferation index (CBPI) for cytotoxicity assessment. An untreated, media only sample was used as the negative control whilst 0.1µM of Aflatoxin B1, a known liver carcinogen, was used as a positive control for genotoxicity. 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). Mean data of three biological replicates (n=3) is presented ± SD.

2. Experimental Section

2.1 Gastrointestinal (GIT) Model

Experiments were performed using monocultures of Caco-2 (DSMZ), HT29-MTX-E12 (previously 'E12'; ECACC via Sigma Aldrich) and THP-1 (ATCC) cells. Information on culture medium composition were included as supplementary information (Table S7).

Table S7 Cell culture medium composition for GIT-related experiments

Cell line	Basis	FBS conc. (%)	Additives
Caco-2	MEM (Gibco, 10370070)	20*	Penicillin/Streptomycin (1%), L-glutamine (1%)
HT29-MTX-E12	DMEM, high glucose (Gibco, 41965039)	10*	Penicillin/Streptomycin (1%), non-essential amino acids (1%)
THP-1	RPMI	10*	Penicillin/Streptomycin (1%), D-glucose (0.6%), sodium pyruvate (1%), mercaptoethanol (500 µL of 50 mM)

*exposure experiments were conducted in 'starvation medium' containing 1% FBS

Table S8 Experimental parameters

Assay	Well format	Seeding density	Volume (µL)	Minimum concentration (µg/cm²)	Maximum concentration (µg/cm²)
Cytokine release	24	$6x10^4$	500	1	80
WST-1	96	$2x10^4$	100	10	80
Comet Assay	24	$6x10^4$ (Caco-2) $1.2x10^5$ (E12)	500	10	80

2.1.1 WST-1 Cell Viability Assay: THP-1 cells were differentiated with PMA (100nM), for 24hrs. The cells were detached with Accutase, re-seeded and left to re-attach for 1hr (**Table S8**). After re-attachment, the supernatant was discarded and the cells exposed to pristine or PT Ag Sigma or amorphous SiO_2 ENM (Table S8) in starvation medium containing 1% FBS. The assay was

performed according to the nanOximet project SOP (https://www.nanopartikel.info/projekte/era-net-siinn/nanoximet/veroeffentlichungen-nanoximet). The absorbance was measured spectrophotometrically (Thermo Scientific, Multiskan Go) at 450 and 630 nm.

2.1.2 Comet Assay: The impact of artificial digestion on Ag Sigma-induced DNA damage was investigated in confluent Caco-2 and E12 monocultures using the alkaline comet assay as described by Thongkam *et al.*,[36] with the following adjustments; the cells were harvested after 24hr exposure. The cell suspension (40µL) was mixed with 240µL low melting point agarose and pipetted (120µL) onto agarose-coated microscopy slides. Microscopy analysis (Olympus BX60) was performed using a U-RFL-T UV-burner at 400x magnification and the Comet Assay IV software. Every sample was prepared in duplicate and 50 nuclei were counted per slide.

2.1.3 ELISA: The cytokine release was quantified by ELISA as described by Kinsner *et al.*[37] Briefly, DuoSet antibody kits for IL-8, IL-6, and TNF-α (DY208; IL-8, DY206; IL-6 & DY210; TNF-α, R&D Systems) were used according to the manufacturer's recommended concentrations. The primary antibody (ab) was incubated in coating buffer (0.1M $NaHCO_3$, pH 8.2) over night at room temperature (RT). The samples were added undiluted (IL-6 and TNF-α) or 1:2-1:10 diluted in 1% BSA/PBS (IL-8). The secondary ab was diluted in 1% BSA/PBS and incubated for 2hrs at RT. After incubation with horse-radish peroxidase, 100µL of BioRad TMB Peroxidase EIA Substrate Kit was added to each well and incubated for 10-20 mins. The reaction was stopped with 50µL 1M H_2SO_4. The absorbance was measured spectrophotometrically at 450 nm (Thermo Scientific, Multiskan Go). The standard curve was plotted as 4-parameter logistic fit.

2.1.4 Statistical Analysis: Statistical analysis for experiments using the GIT model were performed using Prism 8, GraphPad Software, Inc. (USA) to conduct a One-way or Two-way ANOVA and Tukey's post hoc test as specified in the corresponding figure legends.

2.2 3D *In Vitro* Hepatic Spheroid Models

Prior to hepatic spheroid ENM exposure, we employed three sequential incubations. The Ag Sigma ENMs at a concentration of 4.0mg/mL were first PT in IUF_G (pH 2.7) and IUF_I (pH 9.5) as described previously in the experimental section, then transferred to human blood plasma as the third pre-treatment fluid for 1hr to simulate their translocation into the circulatory system prior to reaching the liver. In parallel, 2.56mg/mL of pristine Ag Sigma were dispersed for 16 mins in 0.05% Bovine Serum Albumin (BSA) using the probe sonication (Branson Sonifier 250, Ø 13 mm, 400 W output power, 20 kHz) method described in the NanoGenoTox Dispersion Protocol (Grant Agreement No. 20092101, 2018). Following this, both the PT and pristine Ag Sigma were diluted in cell culture media to the required concentration for exposure to 3D *in vitro* hepatic spheroid models. An acute 24hr exposure scheme was assessed with neat, pristine ENMs and GIT PT ENMs.

Post Ag Sigma exposure, the biochemical endpoints evaluated included liver functionality using the BCG Albumin Assay Kit (MAK124, Sigma, UK), (pro-)inflammatory response using both IL-8 and TNF-α ELISAs (DY208 & DY210 DuoSet ELISA, R&D Systems) and genotoxicity using the Cytokinesis-Block Micronucleus (CBMN) Assay in conjunction with the Cytokinesis-Block Proliferation Index (CPBI) for determining cytotoxicity post-acute ENM exposures. A negative, untreated media control was used alongside two positive chemical controls; 0.1μM of a known liver carcinogen, Aflatoxin B1 (Cat# No: A6636, Sigma Aldrich, UK) as a positive control for genotoxicity and 0.25μg/mL of TNF-α protein (Cat# No: 2-35076- 50μg, BioTechne, UK), an inflammatory inducing agent used as a positive ELISA assay control. The adapted CBMN Assay with different cytotoxicity measures were used as described by Llewellyn *et al*.[18] All experiments were performed with three biological replicates with data presented as the mean ± SD.

2.2.1 Statistical Analysis

Statistical analysis was performed using Prism 8, GraphPad Software, Inc. (USA). Shapiro-Wilk test was used to calculate normality for each data set. For normally distributed data, One-way ANOVA

with Sidak's post hoc were used. For non-parametric data, Kruskal-Wallis test was used to calculate significance when there were more than two variables, with Dunn's multiple comparisons test.