Pre-clinical 2D and 3D Toxicity Response to A Panel of Nanomaterials; Comparative Assessment of NBM-Induced Liver Toxicity

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Abstract

Nanobiomaterials, or NBMs, have been used in medicine and bioimaging for decades, with wide-reaching applications ranging from their uses as carriers of genes and drugs, to acting as sensors and probes. When developing nanomedicine products, it is vitally important to evaluate their safety, ensuring that both biocompatibility and efficacy are achieved so their applications in these areas can be safe and effective. When discussing the safety of nanomedicine in general terms, it is foolish to make generalized statements due to the vast array of different manufactured nanomaterials, formulated from a multitude of different materials, in many shapes and sizes, therefore NBM preclinical screening can be a significant challenge. Outside of their distribution in the various tissues, organs and cells in the body, a key area of interest is the impact of NBMs on the liver. A considerable issue for researchers today is accurately predicting human-specific liver toxicity prior to clinical trials, with hepatotoxicity not only the most cited reasons for withdrawal of approved drugs, but also a primary cause of attrition in pre-launch drug candidates. To date, no simple solution to adequately predict these adverse effects exist prior to entering human experimentation. The limitations of the current preclinical toolkit are believed to be one of the main reasons for this, with questions being raised on the relevance of animal models in pre-clinical assessment, and over the ability of conventional, simplified *in vitro* cell based assays to adequately assess new drug candidates or NBMs. Common 2D cell cultures are unable to adequately represent the functions of 3D tissues and their complex cell–cell and cell–matrix interactions, as well as differences found in diffusion and transport conditions. Therefore, testing NBM toxicity in conventional 2D models may not be an accurate reflection of the actual toxicity these materials impart on the body. One such method of overcoming these issues is the use of 3D cultures, such as cell spheroids, to more accurately assess NBM-tissue interaction. In this study, we introduce a 3D liver model cultured from HepG2 cells to assess both the cytotoxicity and viability observed following treatment with a variety of NBMs, namely a liposome (in the specific technical name = LipImage), a gold nanoparticle (AuNP) and a panel of polymeric (in the specific technical name = PACA) NBMs. This model is also in compliance with the 3Rs policy of reduction, refinement and replacement in animal experimentation [1], and meets the critical need for more advanced *in vitro* models for pre-clinical nanotoxicity assessment.

1. Introduction

Nanoparticles (NPs) are used today for a wide variety of applications ranging from in the fields of textiles and engineering, to cosmetics, food, and specifically medicine, where they are used in diagnostics, biosensing, drug delivery and therapeutics [2, 3]. Due to their far-reaching applications, increasing use and intention that they will be delivered to the human body, nanoparticles used for biomedical applications, or NBMs, must have their potential fates and adverse effects considered and rigorously tested to achieve overall safety and acceptance. NBMs are primarily administered via inhalation, ingestion, dermal administration and IV injection, whereby they are distributed all over the body, before accumulation in key secondary organs such as the spleen and liver [2, 4, 5].

With regard to NBM safety assessment, for decades the gold standard has been a barrage of *in vitro* assays based upon 2D cell cultures, however there is increasing information to suggest that these models do not appropriately mimic human responses and discrepancies can be seen between *in vitro* pre-clinical assessment and *in vivo*. A 3D environment is essential for cells to grow and metabolize correctly [6], with the function and phenotype of individual cells highly dependent on the complex interactions which occur between the 3D structure of the ECM and its neighboring cells [7]. When cultured in two-dimensions however, these complex cell-cell and

cell-matrix interactions are impeded, which has a knock-on effect in recapitulating accurate and predictive *in vivo* cellular responses [8]. Therefore, whilst 2D cell cultures are cheap to use, easy to maintain and work with, and whilst they have provided invaluable information on the cellular responses imparted by drugs and nanobiomaterials (NBMs) for decades, tests which are based upon 2D *in vitro* cell culture models do not accurately predict toxicity of NBMs due to their lack of key physiological processes, including transport of these materials through cells [7, 9]. This is further emphasized in many studies, including one works on the toxicity screening of quantum dots [10], carbon nanotubes [11] and magnetic NPs [12] whereby large cytotoxicity was induced in 2D *in vitro* cell cultures, however this effect was not mimicked in animal models where no adverse effects were observed [13–15].

The large discrepancies between 2D cell culture and animal models is becoming increasingly apparent, and in recent years more emphasis has been placed on advanced *in vitro* methodologies for assessing interactions and possible effects of NBMs [16], due to the fact traditional *in vitro* cell models lack not only the phenotypic details of the *in vivo* environment, but also because physiological function and crosstalk between cells also is highly reductionist in traditional models. 3D models, such as cellular spheroids, have been described as a potential methodology to overcome these issues, bridging the gap between traditional 2D models and *in vivo* animal work, and acting as better predictors of NBM toxicity [17]. Various new mono- and co-culture 3D models have been developed, combining different relevant cell types into one organotypic model which can be used to not only determine NBM toxicity, but also their interactions with various cell types in a 3D environment [16]. Whilst the majority of 3D models are still not standardised or validated methods for assessing the cellular interactions and toxicities associated with NBMs, in recent years many studies have used them in preclinical investigation, for applications in both the optimization of NBM physicochemical properties and screening of their therapeutic effects [18].

Due to the central role the liver plays in the metabolism, clearance and biotransformation of drugs and nanobiomaterials (NBMs), another consideration for researchers today has been predicting human-specific liver toxicity [19]. As a means of reducing both time and cost, over many decades a vast array of *in vitro* methodologies have been developed to test the liver toxicity of NBMs. These models have led to many important insights into toxicity, safety and efficacy of these materials, and they are essential tools in both the discovery and preclinical stages of drug/NBM development [20, 21]. For decades, *in vitro* 2D cultures of the liver have been the gold standard for determining acute hepatotoxicity of NBMs. Currently, 2D *in vitro* assays are based on either primary cell cultures derived directly from harvested liver tissue, or immortalized hepatic cell lines, i.e., primary cells which have been genetically transformed to generate rapidly-proliferating, easily cultured cells with artificial phenotypes that can be grown for prolonged periods. Whilst useful early predictors of toxicity, these immortalized cell lines have reduced metabolic competencies in comparison to their primary hepatocyte counterparts.

Therefore, in recent years, there has been a small push towards using 3D liver spheroid cultures for assessing the impact of NBMs. To date however, the effect of each of the chosen NBMs presented in this study on a HepG2 spheroid model has not yet been discussed in literature, making this work a significant advancement in this area. Here, for the first time, a dye-loaded liposome, a 20 nm PEGylated AuNP, and three PACA NBM formulations (i.e., unloaded, dye-loaded and drug (CBZ) loaded) were characterized, before their ability to impact HepG2 cell and spheroid viability and their ability to induce cytotoxicity was assessed in both models.

2. Materials And Methods

2.1 Cultivation of HepG2 Cells and preparation of HepG2 spheroids

Human liver hepatocellular carcinoma (HepG2) cells were provided by SINTEF, Norway. HepG2 cells and 3D hepatic spheroids were grown in low glucose Dulbecco's Modified Eagles Medium, supplemented with 10% foetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Gibco, Invitrogen Ltd, VWR), and maintained at 37°C and 5% CO₂. For all experiments, HepG2 cell passage number was restricted between ten and twenty. At 80% confluence, cells were detached from T75 flask (Thermo Fisher, Ireland) using TrypLE™ (Gibco, Invitrogen, Oregon, USA), centrifuged, and resuspended in 1 mL culture medium. Cells were counted using a Countess automated cell counter (Thermo Fisher, Ireland) and seeded in either 2D or 3D environments in an appropriate manner for the experimental endpoint under investigation.

For 2D monolayers, 10,000 HepG2 cells were seeded on 96-well plates (Thermo Fisher, Ireland). Cells were left to adhere for 24 h before being treated with chosen NBMs for desired timepoints. For HepG2 spheroid formation, cells were seeded in CellStar® 96 well ultra low attachment (ULA) cell repellent plates (Grenier, BioOne, UK) (Figure 1) at a density of 1000 cells per well in 100 µl medium (Figure 1). After three days of incubation at 37°C with 5% CO₂, medium was changed with care taken not to disturb spheroid. After 1 week, spheroids had an approximate diameter of 300 µm and were taken for further analysis or exposed to NBMs (both detailed below).

2.2 NBM preparation

Five NBMs were used in this study: 20 nm PEGylated AuNP (purchased from nanoComposix, San Diego, California); LipImage™815, a liposome encapsulating IR780 dye (kindly supplied by CEA-LETI, France); Three polymeric nanoparticles all provided by SINTEF (Trondheim, Norway), i.e., PACA, an unloaded polymeric nanoparticle, PACA loaded with NR668-dye and CBZ-loaded PACA. A nanoparticle control, 10 µg/mL TiO₂ (NM101 supplied by JRC, Italy), in both static and dynamic conditions, was also included in study design (Figure 9). A concentration of 10 µg/mL was chosen as control as it kills <50% cells.

The AuNP were supplied at a mass concentration of 1.00 mg/ml and a molar particle concentration of 2.5×10^8 particles (mol/l). Particles were 20 nm in size. Dispersion media used was milliQ water. The AuNP was deemed sterile and endotoxin free, i.e., BioPure™, with an endotoxin quantity of <5 EU/ml (within acceptance criteria). AuNP was negatively charged, with a zeta potential of -24 mV. LipImage, the IR780 dye-loaded liposome, was provided at a particle concentration of 95 mg/ml (9.5%) and a dye concentration (HPLC) of 239.5 µM (252 µM/100 mg particle). LipImage has a diameter of 80 nm. Dispersion media used was 154 mM NaCl and ascorbic acid (1.75 g/l). LipImage was deemed sterile and endotoxin free, with an endotoxin quantity of < 1 UI/ml (within acceptable amount).

PACA nanoparticles were synthesized under aseptic conditions at SINTEF mini-emulsion polymerization. Prior to synthesis, all solutions were sterile filtered, and all equipment was autoclaved. An oil phase consisting of poly(ethylbutyl cyanoacrylate) (PEBCA) (Quantum Medical Cosmetics) containing 2% wt Miglyol 812 (Cremer) and 10 wt% vanillin was prepared. For drug-loaded particles, the oil phase was added 12 wt% Cabazitaxel (BioChemPartner) and only 2 wt% vanillin was used. For dye-loaded particles, the oil phase was added either 0.4 wt% IR-780-Oleyl (custom synthesis at CEA LETI) or NR668 (modified Nile Red, custom synthesis [22] at SINTEF). An aqueous phase consisting of 0.1 M HCl containing the two PEG stabilisers (Brij®L23 and Kolliphor®HS15,

both Sigma-Aldrich, 5 wt% of each) was added to the oil phase. The water and oil phases were mixed and immediately sonicated for 3 min on ice (6 × 30 s intervals, 60% amplitude, Branson Ultrasonics digital sonifier). The solution was rotated (15 rpm) at a room temperature overnight. The pH was then adjusted to 5.0 to allow further polymerisation for 5 h at room temperature. The dispersions were dialyzed (Spectra/Por dialysis membrane MWCO 100.000 Da) against 1 mM HCl to remove unreacted PEG. The size (z-average), polydispersity index (PDI) and the zeta potential of the NPs in phosphate buffer (10 mM, pH 7.0) were measured by dynamic light scattering (DLS) and laser Doppler Micro-electrophoresis using a Zetasizer Nano ZS (Malvern Instruments). To calculate the amount of encapsulated drug, the drug was extracted from the particles by dissolving them in acetone (1:10) and quantified by liquid chromatography coupled to mass spectrometry (LC-MS/MS) using an Agilent 1290 HPLC system coupled to an Agilent 6490 triple quadrupole mass spectrometer.

2.3 Hydrodynamic Diameter and Zeta Potential Measurements of the NPs

All samples were stored at 4°C and equilibrated to RT before characterization, which was kindly undertaken in house by Gabriele Vella, LBCAM, TCD. For nanoparticle tracking analysis (NTA), the NS500 NanoSight (Malvern Panalytical, UK) along with the Nanosight 3.2 software package (NTA build 3.2.16) following the European Union Nanomedicine Characterisation Laboratory (EUNCL) approved protocol was used [23]. NBMs were prepared and diluted between 1:5000 and 1:100,000 using D-PBS (-MgCl₂ and CaCl₂). A 405 nm laser was used to visualise particles present in a given field of view. 60 s recordings of the laser interacting with particles are captured using an EM-CCD camera. The camera level and focus were manually controlled and chosen by the operator (Camera level = 10 for the 1:5,000 and 1:10,000 dilutions. Camera Level = 13 for the 1:100,000 dilution). The detection level was chosen by the operator (Detection Level = 3 in all dilutions) and the recordings were subsequently analysed by the Nanosight 3.2 software to determine particle numbers per frame and sample concentrations. Through the phenomenon of Brownian motion, the particle size can be determined by the software. The D-PBS used in the dilution of NBMs was also analysed to assess background particle levels. 30 nm gold citrate nanoparticles of known size were used as reference materials for the Nanosight.

For dynamic light scattering (DLS), NBMs analysed using a Zetasizer Nano ZS system (Malvern UK), running Zetasizer version 7.13, using the EUNCL approved -PCC-001 SOP 'Measuring Batch Mode DLS' [23] 1:100 dilution of the sample for DLS was made up in Dulbecco's Phosphate-Buffered Saline (D-PBS) buffer (-MgCl₂ and CaCl₂). Samples were pipetted to ensure adequate mixing. Samples were loaded into a DTS0012 disposable cuvette and was subject to a 300 second equilibration time as per the EUNCL SOP. A total of twelve x 10 second runs per measurement were recorded for the sample and was subject to 10 measurements with a zero second delay between measurements. The backscatter angle (173° NIBS Default) was used in the analysis, with optimum positioning enabled. Automatic attenuation selection was enabled, and the General-Purpose Analysis Mode was chosen.

2.4 Morphological assessment of HepG2 spheroids

Brightfield microscopy was used to assess the growth, morphology and size of hepatic spheroids across culture period. Spheroids were imaged using an epifluorescent microscope (Nikon TE300, equipped with 10X objective and QCapture Software QImaging). All image processing and measurements were undertaken using ImageJ software.

Histological analysis was used to assess the internal structure of hepatic spheroids. Cultures were washed with PBS and fixed for 1 h in 4% PFA, before being embedded in 2% EEO agarose in PBS in plastic molds. Agarose blocks were dehydrated in increasing concentrations of EtOH (20%, 40%, 60% EtOH in H₂O), and paraffin embedded using a tissue processor with the help of Dr Gavin McManus (Trinity Biomedical Sciences Institute, Trinity College Dublin). Paraffin blocks were sectioned using a microtome and sections stained with Haematoxylin and Eosin (H&E). Briefly, spheroid section slides were de-waxed and rehydrated by passing them through solvents in the following manner: Xylene 5 min, Xylene 4 min, 99% IMS 3 min, 90% IMS 2 min, 70% IMS 1 min, before running them in cold water for 1 min. Slides were then placed in Haematoxylin for 5 mins before being rinsed in running tap water for 5 min. Slides were dipped in acid alcohol (1%) for 2 seconds, rinsed in tap water for 30 seconds, before staining with Eosin (5 min). Slides were briefly rinsed in tap water again, with care taken to not remove Eosin. Sections were then dehydrated and cleared in the following manner: 70% IMS 1 min, 90% IMS 1 min, 99% IMS, 2 min, Xylene 3 min, Xylene 3 min. Slides were mounted using DPX, with cover slide added at a 45° angle. Stained tissue sections were then imaged by epifluorescent microscopy (Nikon TE300, equipped with 10X objective and QCapture Software QImaging). Image analysis was performed by ImageJ software.

2.5 Quantification of cell number in spheroids

Total cell number in HepG2 spheroids was determined by adding TrypLE™ to spheroids (3 pooled spheroids) and mechanically dissociating by vigorous pipetting, staining with 1:1 trypan-blue, and counting cells using the Countess™ automated cell counter (Thermo Fisher, Ireland).

2.6 Sensitivity to hepatotoxins

Sensitivity of spheroid cultures to a panel of common hepatotoxins was tested using a protocol detailed previously in works from Gaskell *et al.* [24] and Bell *et al.* [25], and using information listed in the MIP-DILI training set [26]. The hepatotoxic compounds included: acetaminophen, diclofenac, trovafloxacin and fialuridine. HepG2 spheroids grown for 3 days and 10 days were exposed to the hepatotoxic compounds diluted in cell culture medium, with an addition dose administered 2-day after the first exposure. Concentrations tested for each compound were:

Acetaminophen: 10000, 5000, 1250, 625, 300, 150, 75 µg/ml

Diclofenac: 400, 200, 100, 50, 25, 12.5, 6.25 µg/ml

Trovafloxacin: 500, 200, 100, 50, 25, 12.5, 6.25 µg/ml

Fialuridine: 200, 100, 50, 25, 12.5, 6.25, 3.15 µg/ml

Viability of the treated cultures was determined using the Cell Titer-Glo® assay (ThermoFisher, Ireland) as per manufactures protocol. Culture media alone with Cell Titer-Glo® reagent was used as a blank control and subtracted from all sample values. Cell viability was calculated as a percentage of untreated vehicle control samples. A dose-dependent response curve was plotted, and estimations were made about what concentration of drug would cause a 50% reduction in cell viability (IC₅₀ value). A dose-response curve was plotted and IC₅₀ values determined from the curve. Experiments were run in triplicate (n=3).

2.7 Exposure to NBMs

2D and 3D cultures were exposed to AuNP, LipImage or various PACAs, using a variety of concentrations detailed below in Table 1:

Table 1
NBMs used in this study and the concentrations used

Nanobiomaterial	Exposure (µg/mL; 30 min, 3 h, 24 h)
AuNP	1, 5, 10, 20, 30, 50
LipImage	10, 50, 100, 200, 500, 1000
PACA	1, 5, 10, 20, 32, 64, 128, 256, 512, 1024
CBZ-loaded PACA	32, 64, 128, 256, 512, 1024
NR668-loaded PACA	32, 64, 128, 256, 512, 1024

Stock NBM suspensions were vortexed and appropriate dilutions made in cell culture medium. For NBM exposures, briefly, cell culture medium was removed from wells of culture plates and wells washed with pre-warmed PBS. For ATP quantification and LDH assay: 100 µl of each NBM concentration diluted in culture medium was added to each well for three incubation/exposure timepoints, 24, 48 and 72 hours.

2.8 Viability and cytotoxicity assessment via CellTiter-Glo® and LDH Assays

Experimental workflow for viability and cytotoxicity assays is detailed in Figure 2.

The CellTiter-Glo® Luminescent Cell Viability Assay is a method of assessing cell viability in cultures based on the quantitation of ATP present, an indicator of metabolically active cells. Cultures were washed with pre-warmed PBS and ATP content quantified using Cell Titer-Glo® (Promega Corporation, MyBio Ltd, Ireland), performed as per manufacturers protocol. All samples were tested in triplicate ($n_{\text{replicates}} = 3$), and experiments were repeated three times ($n_{\text{tests}} = 3$). Data are presented as average $\pm$ standard deviation of the mean. Assays and experimental controls were included in the experimental design. Untreated controls where in the forms of no NBMs, only culture medium. Experimental positive control, at 125 µM Valinomycin was chosen as this concentration caused <50% maximal cell death after 24 h. Assay positive control was 10x lysis buffer, as from supplier. NBM positive control was 10 µg/mL TiO₂ as this concentration caused <50% maximal cell death after 24 h when studied in both static and dynamic conditions, as shown in Figure 9.

The LDH assay is a cytotoxicity assay which is used to assess the level of plasma membrane damage in a cell population. Supernatants were harvested from 2D cultures/3D monoculture and multicellular spheroids exposed to NBMs at three different incubation/exposure timepoints, 24-, 48- and 72- hours. LDH release in the supernatants (an indirect measure of cytotoxicity responses) was assessed using the Thermo Scientific Pierce LDH Cytotoxicity Assay Kit (Fisher Scientific, Ireland), following manufacturers' protocols. Untreated cultures and 2D cultures/3D spheroids exposed to LDH Lysis Buffer (1X in supplemented culture medium; 45 min; 37°C) were included in the experimental design as negative (NT) and Positive (PT) controls, respectively. Absorbance values at wavelengths (λ) equal to 490 and 680 nm were read using an Epoch microplate reader (Biotek, Mason

Technologies, Ireland). The LDH activity was calculated using Equation (4.1); whereas the percentage (%) cytotoxicity was extrapolated from Equation (4.2).

$$\text{LDH activity} = \text{Absorbance } \lambda=490\text{nm} - \text{Absorbance } \lambda=680\text{nm} \quad (\text{Equation 4.1})$$

$$\text{Cytotoxicity (\%)} = \frac{\text{LDH activity Treated culture} - \text{LDH activity (NT)}}{\text{LDH activity PT} - \text{LDH activity (NT)}} \quad (\text{Equation 4.2})$$

All samples were tested in triplicate ($n_{\text{replicates}} = 3$), and experiments were repeated three times ($n_{\text{tests}} = 3$). Data are presented as average $\pm$ standard error of the mean (SEM).

2.9 Statistical Analysis

Results are presented as mean with standard deviation (SD) of 3 independent experiments ($n = 3$), unless otherwise mentioned. Statistical analysis by Two-way ANOVA, with post hoc Sidak test. All graphs and statistical analysis were undertaken using GraphPad Prism 9, Version 9.4.0 (GraphPad Software, Inc, San Diego, USA). p-values are marked by * as $p < 0.05$, ** as $p < 0.01$, *** as $p < 0.001$ and **** as $p < 0.0001$.

3. Results

3.1 Characterization of NBMs - Hydrodynamic Diameter and Zeta Potential

Five different NBMs were used in this study as listed above.

It is widely known that both size and shape greatly influence NBM-induced toxicity. The potential of an NBM formulation to induce cellular damage is directly dependent on its size. Therefore, each nano-formulation used in this study was carefully characterized using conventional nano-characterization techniques. NMB characterization for each material can be viewed in an article in this issue from Tutty *et al.* [27].

3.2 Brightfield microscopy and H&E analysis of spheroids

Spheroid diameter gradually increased over the culture period, with spheroids appearing generally spherical and uniform in shape (Supplementary Figure 1). Loose spheroids were formed after 1 d, which further compacted to form fully intact spheroids after 3 d. Spheroids gradually increased in diameter over the culture period, with loss of circular morphology after 19 d. A seeding concentration of 1,000 cells per 100 μl culture medium yielded uniform, circular spheroids, with consistent morphology overtime and a diameter of $294.26 \pm 10.43 \mu\text{m}$ at 3 d and of $647.95 \pm 16.86 \mu\text{m}$ at 31 d. At 7 days, the chosen timepoint for any further experiments, spheroids had defined perimeters, limited cell death and compact structure (Figure 3).

To assess changes in internal spheroid structure and in morphology, HepG2 spheroids were paraffin-embedded, sectioned and stained with H&E. Spheroids formed from a cell seeding concentration of 1,000 cells per 100 μl culture medium, confirmed a compact, uniform internal structure with defined outer perimeters, and direct cell-cell contacts similar to those observed in human liver. Limited apoptosis and no necrotic core are visible up to 27 d (Supplementary Figure 2), when a small patch of necrosis could be observed in spheroid core. At 7 days, the chosen timepoint for any further experiments, spheroids were healthy and limited cell death is visible (Figure 4).

3.3 Cell count in HepG2 spheroids

The increase in spheroid diameter observed from bright field microscopy was reflected in an increase growth as cell number overtime (Figure 5). The cell number increased as culture time progressed, with a sigmoidal pattern observed. A plateau was reached after approximately 19 d, indicating a reduced proliferation and senescence of HepG2 cells within the spheroids.

3.4 Ability of spheroids to detect hepatotoxicity - Sensitivity of HepG2 spheroids to human hepatotoxins and comparison to 2D monolayers

Spheroids and HepG2 monolayers were treated with varying concentrations of four common hepatotoxic compounds namely, acetaminophen, diclofenac, fialuridine and trovafloxacin. Following a protocol detailed in Gaskell *et al*, a 4-day repeat-dosing regimen was used to expose 3D spheroids. For 2D monolayers, however, acute dosing only was assessed as the monolayers became over-confluent and exhibited reduced viability after 48 h in culture. After treatment with the hepatotoxic compounds, a dose-dependent reduction in ATP production was observed in spheroids (Figure 6). Each of the four chosen compounds was more toxic in 3D spheroids then in 2D monocultures, although differences were not statistically significant. The concentration causing a 50% reduction in cell viability (IC₅₀), was estimated for each drug tested from the graphs, and is reported in Table 2. A comparison to data extracted from the scientific literature is also reported.

Table 2

Sensitivity of HepG2 spheroids and monolayers to a panel of common hepatotoxins. HepG2 cells and spheroids were treated with varying concentrations of common hepatotoxins. Viability was assessed using ATP quantification and concentration of drug to cause a 50% reduction in viability was estimated from assay results, with comparisons made to literature. PHH denoted primary human hepatocytes.

Compound	Value at which 50% reduction in viability is observed (µM)				Ref
	HepG2 spheroids - 4-day repeat dosing	HepG2 2D monolayer - 24 h acute dosing	2D monolayer of HepG2 cells - Data from the scientific literature	2D monolayer of (PHH) - Data from the scientific literature	
Fialuridine	>200	>200	>200	>1000	[17, 18]
Trovafloxacin	>100	>400	>1000	~200	[17]
Acetaminophen	>8000	>10000	22800	~20000	[17]
Diclofenac	>250	>400	763	331	[17, 19]

Size of spheroids or their duration in culture was also found to play no part in their responses to hepatotoxins, with no significant differences found when the same experiment was repeated on spheroids cultured for 10 d, with repeated dosing undertaken at day 12 (Supplementary).

3.5 Viability of 2D and 3D liver models following treatment with NBMs

The effects of AuNP, LipImage and each PACA NBMs on the viability of HepG2 cells in 2D and 3D cultures were measured after 24 h, 48 h and 72 h exposure, using the Cell Titer-Glo® luminescent viability assay (Figure 7). No significant reduction in viability in 2D and 3D cultures from AuNP and LipImage (Figure 7A and B). Significant differences in cell viability were observed however between both culture types following treatment with all PACA formulations, most notably as concentrations increased. The largest reduction in viability in 2D culture was seen with CBZ PACA (Figure 7E).

3.1.6 Cytotoxicity induced by NBMs in 2D and 3D liver models

Following on from the assessment of cell viability, the toxic effects of each NBM was quantitatively assessed using the LDH assay. Here, the amount of LDH reagent that leaks out from the plasma membrane of damaged cells is detected. As determined by both 2D and 3D culture supernatants, no significant cytotoxicity was observed in 2D and 3D cultures following exposure to both AuNP and LipImage (Figure 8A and B). Significant dose-dependent cytotoxicity was observed in all PACA samples, most specifically for CBZ-PACA cultures and in particular at larger concentrations (Figure 8C, D and E).

4. Discussion

In this study, a 3D hepatic cell culture model was introduced, characterized and then used for the testing of a variety of NBM formulations. Whilst the current gold-standard for drug metabolism and toxicity studies is the use of primary human hepatocytes (PHH) [28, 29], their short life span, high costs, limited availability and the inter-donor variation observed significantly limits their use in high-throughput *in vitro* toxicity screening. For this reason, the immortalised hepatocyte cell line HepG2 was used for this work. Although, when cultured in 2D, HepG2 cells exhibit many limitations including loss of liver-specific structure and functionality [28, 30], their ease-of-use as compared to PHH, coupled with a stable phenotype and no donor variation [28, 31], offer many advantage for the development of standard operating procedures (SOPs) for preclinical screening applications. Furthermore, it has been shown that cultured in 3D environments, HepG2 cells regain some of the characteristics they lose when cultured in 2D. In 3D, HepG2 cells exhibit reduced proliferation rates, self-organise and differentiate to form 3D spheroids, which regain lost hepatic structure and function.

For this study, HepG2 spheroids were cultured in CellStar® cell repellent plates and characterised, before being used in the assessment of five chosen NBMs. These plates were chosen as they are easy to acquire, standardised and high throughput in nature, whilst also known to produce reproducible spheroids [32–34]. HepG2 spheroids formed successfully using this method, with the cells in each well aggregating to form a single spheroid. Spheroids formed from this method could be rapidly cultured by directly seeding into the plate, without the need for complicated transferring of spheroids or expensive hanging drop plates, or the need for much optimisation, and they were cost-effective and demonstrated a higher degree of reproducibility in forming 3D HepG2 spheroids. As presented in Figure 5 (Brightfield) and Figure 6 (H&E), spheroids formed from a cell seeding concentration of 1,000 cells per 100 µl culture medium and cultured for 7 days, the chosen timepoint for further experimentation, spheroids had defined perimeters, limited cell death and compact structure.

One of the major uses of hepatic spheroids in pre-clinical research and one of their most exciting and useful applications today is in their ability to predict hepatotoxicity of various drugs and materials. To probe whether HepG2 spheroids would be better predictors of hepatotoxicity than 2D cultures, spheroids and HepG2 monolayers were treated with varying concentrations of four common hepatotoxic compounds; namely acetaminophen, diclofenac, fialuridine and trovafloxacin (information on these compounds and how they impact the liver is listed in supplementary). Following a protocol detailed in Gaskell *et al*, a 4-day repeat-dosing regimen was used to expose 3D spheroids. For 2D monolayers, however, acute dosing only was assessed as the monolayers became over-confluent and exhibited reduced viability after 48 h in culture. After treatment with the hepatotoxic compounds, a dose-dependent reduction in ATP production was observed in spheroids (Figure 10). Each of the five chosen compounds was found more toxic in 3D spheroids compared to the 2D monocultures, although differences were not statistically significant. The concentration causing a 50% reduction in cell viability (IC_{50}), was estimated for each drug tested from the graphs and is reported in Table 5. A comparison to data extracted from the scientific literature is also reported in Table 2. Each of the four hepatotoxic compounds screened in this study exhibited more toxicity and greater sensitivity in HepG2 spheroids when compared to the 2D monolayer, with spheroid culture time was also found to have no effect on this result with spheroids cultured for both 5 and 12 days exhibiting no significant differences in sensitivity (Supplementary Figure 3). It can be hypothesised that this increased toxicity is due to the direct cell-cell contacts, increased liver-specific functionality and structure of the HepG2 spheroids allowing the hepatotoxins to exert their effects, in comparison to the 2D monolayers, with their limited monolayer planar cell contacts and lack of transporter functionality. Several other studies have also found that 3D hepatic spheroids are greater predictors of hepatotoxicity than 2D monolayers, supporting the results in this study and the hypothesis that culturing hepatic cells as 3D spheroids increases hepatotoxin sensitivity [35–37].

Finally, the impact five NBMs had on cell viability and any induction of cytotoxicity was assessed up to 72-hour exposure. To overcome some of the key issues seen with the pre-clinical assessment of NBMs, such as interference with assays and/or reagents, in this study the CellTiter-Glo® ATP quantification, a frequently used assay for 3D microtissues, was used for determining 2D and 3D spheroid viability. Here, treated spheroids are incubated with CellTiter-Glo® reagent and viability is judged by the quantification of a luminescent signal formed when luciferin is converted by luciferase, which is in turn indicative of cytoplasmic ATP concentration. This reagent successfully penetrates even large spheroids and has an increase lytic capacity, allowing the time effective, standardised and more accurate determination of viability in 3D structures when compared to other standard methods [42]. In parallel to assessing viability using ATP quantification, the LDH assay was used to determine NBM-induced cytotoxicity. As the LDH assay is supernatant based, with the cytosolic enzyme LDH released into culture supernatants by compromised plasma membranes, this assay avoids issues with having to dissociate the spheroid structure as is necessary for other membrane integrity assessment methods like neutral red or trypan blue. When assessed using these assays, no reduction of viability or induction of cytotoxicity was observed following treatments with both AuNP and Liplmage in both culture types. This is in agreement with various studies who have shown that similar sized/coated AuNPs and Liplmage are generally biocompatible and well tolerated [43]. In comparison to this, each PACA NBM induced a dose-dependent reduction in viability and increase in cytotoxicity at all time points, most notable after 72 hours, in 2D cultures. A similar toxicity profile was observed in a previous published work from Sulheim *et al*. [44]. The same pattern was not observed in 3D cultures, with significant differences found between both culture types. It can be said that this result is observed and recorded, due to the more *in vivo*-like phenotype of the 3D spheroids and not due to the material type, as

determined by TiO₂ control. The most toxic NBM was found to be the NR668-loaded PACA, with an increase in cytotoxicity and a reduction in viability also observed in 3D cultures for this NBM.

As traditional 2D cell culture models do not adequately represent the structure or the function of 3D hepatic tissues, which have extensive and complex cell-cell and cell-matrix interactions, in combination with vastly different diffusion and transport condition, viability and cytotoxicity assessment of NBM treated liver cultures in two-dimensions, does not accurately reflect the actual toxicity of NBMs in the body. Therefore, the development of advanced 3D liver models for the toxicity screening of these materials *in vitro* is vitally important in order to have more *in vivo*-like, realistic models that have tissue like physiology, for their risk-assessment. Whilst hepatic spheroids have become more popular in recent decades for assessing issues like drug-induced liver injury (DILI), their uses in the study and pre-clinical assessment of NBMs is still in its infancy. In the past couple of years, and in particular in 2020, various publications have acknowledged the potential they hold for accurately assessing nanomaterials in the preclinical *in vitro* evaluation stage. Publications from Dubiak-Szepietowski *et al.* (2016) [45], Elje *et al.* (2020) [46, 47] and Fleddermann *et al.* (2019)[48] have all utilised hepatic spheroids for assessing distribution or toxicity of NPs. These materials however are ones which may be encountered occupationally and are not technically NBMs which can be applied to medical technology, like the materials presented in this study. To date, very few publications have used 3D liver models for assessing toxicity of NBMs, with most studies utilising spheroids to assess depth and penetrations of NBMs in 3D [49–53], and to the best of our knowledge this study *in vitro* toxicity screening, making the work presented in this work a significant advance in the field. In this study, a 3D hepatic cell culture model was introduced and its applicability to the preclinical assessment of NBMs tested. Whilst two materials exhibited no significant differences between the two culture types, due to their overall biocompatibility, great differences were seen between 2D and 3D following treatment with the PACA NBMs, illustrating the importance of testing NBMs in more than one culture setting. With all this being said, the HepG2 3D spheroids presented in this work can be described as a promising and human-relevant model, amenable for the accurate and sensitive screening of NBMs *in vitro*, whilst also being compliant with the 3Rs policy to reduce *in vivo* animal testing. As HepG2 spheroids remain viable for long periods, they are also amenable for repeated or chronic dosing regimens, with the possibility to analyse both sub-acute and delayed toxic effects. Overall, the work presented is a positive contribution to the development of advanced *in vitro* models for screening the safety of nanobiomaterials.

Declarations

Ethics approval

N/A

Consent for publication

N/A

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, [MAT], on special request.

Competing Interests

MAT, APM and GV declare they have no competing financial interests.

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Authors' contribution

MAT and APM conceptualised and developed the experimental work, MAT managed the experimental work with partners. GV carried out the experimental characterisation of the NBMs.

MAT, GV, and APM contributed to the drafting and finalization of this manuscript.

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Figures

Figure 1

Schematic of HepG2 spheroid formation in CellStar® cell repellent plates. (A) Single cells are seeding into non-adherent round bottom wells. (B) After 6 h cells cluster together to form cell spheroids.

Figure 2

Experimental workflow for assessing NBMs hepatotoxicity *in vitro* by measuring changes in viability and cytotoxicity following multiple endpoint exposure.

Figure 3

Brightfield image of a HepG2 spheroid cultured for 7 days.

Figure 4

H&E stained HepG2 spheroid cultured for 7 days. Healthy internal structure is observed with no apoptosis or necrosis observed.

Figure 5

Cell number in HepG2 spheroids grown in CellStar® cell repellent plates. Spheroids were formed starting from a seeding concentration of 1,000 HepG2 cells per 100 µl culture medium and cultured for 31 d. Data shown as mean $\pm$ SEM ($n_{\text{replicates}} = 6$).

Figure 6

Changes in cell viability in 2D monolayers and 3D spheroids of HepG2 cells following exposure to four common hepatotoxins. Spheroids and 2D monolayers were treated with four hepatotoxic compounds, namely (A) fialuridine, (B) trovafloxacin, (C) acetaminophen and (D) diclofenac. Viability following treatments was determined as a function of ATP production and plotted as a percentage of the ATP present in the untreated control. Data represented as mean $\pm$ SEM ($n_{\text{replicates}}=3$). The symbol (**) indicates $p < 0.01$ (multiple t tests with Bonferroni Dunn method).

Figure 7

Evaluation of cell viability in NBM treated hepatic 2D monocultures and 3D hepatic spheroids at 3 different exposure time points using the CellTiter-Glo® ATP quantification assay. (A) AuNP, (B) LipImage, (C) PACA, (D) NR668-loaded PACA and (E) CBZ-loaded PACA all assessed after 24-, 48- and 72- hour exposure. Data is expressed as fraction of viable cells; Data normalized to 1.0 for Positive control (PT – untreated cells) and representative of a minimum of three independent experiments (n=3) and expressed as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Statistical analysis by Two-way ANOVA, with post hoc Sidak test. All graphs and statistical analysis were undertaken using GraphPad Prism 9, Version 9.4.0 (GraphPad Software, Inc, San Diego, USA).

Figure 8

Evaluation of cytotoxicity induced by NBMs in hepatic 2D monocultures and 3D hepatic spheroids at 3 different exposure time points using the LDH cytotoxicity assay. (A) AuNP, (B) LiplImage, (C) PACA, (D) NR668-loaded PACA and (E) CBZ-loaded PACA all assessed after 24-, 48- and 72- hour exposure. Data is expressed as fraction of cells lysed using lysis buffer (1.0 = 100%; PT) and representative of a minimum of three independent experiments (n=3) and expressed as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Statistical analysis by Two-way ANOVA, with post hoc Sidak test. All graphs and statistical analysis were undertaken using GraphPad Prism 9, Version 9.4.0 (GraphPad Software, Inc, San Diego, USA).

Figure 9

Cytotoxicity assessment of control samples in both static and dynamic conditions after 24 hours using the LDH cytotoxicity assay. NT represents no treatment (cell culture medium only), TiO₂ represents 10 μ g/mL nanoparticle positive control tested in both static conditions, and dynamic conditions using a Mimetas rocker, Valinomycin represents a 125 μ M Valinomycin experimental positive control and PT represents 50 μ l 10x lysis buffer assay positive control provided with LDH kit. Data is expressed as fraction of lysed cells (1.0 = 100%; PT) and representative of a minimum of three independent experiments (n=3) and expressed as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Statistical analysis by Two-way ANOVA, with post hoc Sidak test. All graphs and statistical analysis were undertaken using GraphPad Prism 9, Version 9.4.0 (GraphPad Software, Inc, San Diego, USA).

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