

Three-dimensional (3D) hepatic cell culture models to improve the clinical translation of nanobiomaterials (NBMs)



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Submitted by

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DECLARATION

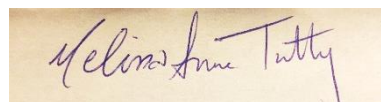
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Melissa Anne Tutty

This research was carried out in the Nanomedicine and Molecular Imaging Group,
Department of Clinical Medicine, Trinity Translational Medicine Institute, St James
Hospital, Dublin 8.

Summary

The main aim of this Ph.D. project is to determine whether hepatic spheroids can be applied to the pre-clinical assessment of nanobiomaterials (NBMs), with particular emphasis on predicting human hepatotoxicity.

Chapter 1 introduces the definition of nanoparticles and nanobiomaterials (NBMs), illustrates their current uses in the clinic, and the safety considerations surrounding their clinical application. The pitfalls associated with the preclinical assessment cascade currently used for NBMs are presented, with an emphasis on the need to shift towards 3D *in vitro* models, particularly when assessing human liver toxicity. This chapter also includes a section describing the hypothesis and aims of this Ph.D. project.

Chapter 2 presents the development and characterization of a hepatic spheroid model formed by human hepatocarcinoma (HepG2) cells. This chapter ends with the expansion of the spheroid model to a multicellular model, with the incorporation of the non-parenchymal (NPC) cell line LX-2 (hepatic stellate cells, or HSCs). The key conclusion from this chapter is that HepG2 cells cultured in a non-adherent environment form uniform, reproducible single spheroids that are structurally and functionally similar to the *in vivo* human liver. Such spheroids remain viable for up to a month and show enhanced sensitivity to hepatotoxins when compared to 2D monolayer cultures.

Chapter 3 reports the characterization of the NBMs chosen for this Ph.D. project; a benchmark 20nm AuNP (representative of a metallic NBM), an IR780 dye-loaded liposome (LipImage™815) and a NR668 dye-loaded polymeric PEBCA NBM (PACA). NBM distribution in 3D hepatic spheroids is qualitatively described as a proof of principle pilot experiment to show NBMs can successfully penetrate the hepatic spheroids and be imaged. , Assessing this was vitally important prior to undertaking the *in vitro* experimentation to evaluate the NBM hepatotoxicity, which is described in Chapter 4. The key conclusion from this chapter is that the NBMs tested can be successfully internalized and imaged in hepatic 3D spheroids and localise within the cell cytoplasm.

Chapter 4 describes the application of 3D hepatic spheroids to the pre-clinical assessment of NBMs. The safety profiles of the three NBMs (metallic NBM, liposome and polymeric NBM) is investigated in the monoculture and multicellular hepatic spheroids developed in Chapter 2, by adapting various assays and techniques detailed in the European Nanomedicine Characterization Laboratory (EUNCL) assay cascade and existing OECD

guidelines. This work resulted in the development of a Standard Operating Procedure (SOP), described within this chapter, detailing how the alkaline comet assay can be used in combination with 3D liver spheroids in order to determine NBM-induced genotoxicity. Notably, this SOP has been rigorously tested and successfully validated by external collaborators and has been accepted as an EU-NCL standard for assessing NBM-induced genotoxicity.

Chapter 5 closes this thesis, describing the overall conclusions of the project and the lessons learned. Future work that will follow on from this research is also discussed

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3. Singh, B., Abdelgawad, M., ; Ali, Z., Bailey, J., Budyn, E., Civita, P., Clift, M., Connelly, J., Constant, S., Hittinger, M., Kandarova, H., Kearns, V., Kiuru, T., Kostrzewski, T., Kress, S., Marsh Durban, V., Lehr, C M., McMillan, H., Metz, J., Monteban, V., Movia, D., Neto, C., Owen, C., Paasonen, L., Palmer, K., Pilkington, G., Pilkington, K., Prina-Mello, A., Roper, C., Sheard, J., Roy, I., Smith, S., Turner, J., **Tutty, M.**, Velliou, E., Wilkinson, J. ‘Towards more predictive, physiological, and animal free *in vitro* models: Advances in Cell and Tissue Culture 2020 Conference Proceedings’, <https://doi.org/10.1177/02611929211025006>, Alternatives to Laboratory Animals, SAGE Journals July 2021.

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SOP

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Credited courses for professional development

PhD Skills Module, 5 ECTS

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Abstract

Despite the wide-reaching applications of nanoparticles that may be used for medical applications, i.e. nanobiomaterials or NBMs, in human health and medicine, their translation to the clinic is staggering slow, with a major issue being unintended liver accumulation and toxicity. *In vitro* 2D cell-based assays and *in vivo* testing are the most popular and widely used methods for assessing liver toxicity at preclinical stages; however, these methods have many limitations in assessing NBM hepatotoxicity. One potential way of overcoming this is by adopting more human-relevant models, such as *in vitro* 3D hepatic spheroids. These have shown great promise in detecting, in a predictive manner, the effects of various hepatotoxins in humans, thanks to their enhanced human-specific structure and functionality when compared to conventional 2D monocultures.

The aim of this Ph.D. project is to assess whether hepatic mono- and multi-cellular 3D spheroids can be successfully applied to the pre-clinical assessment of nanobiomaterials (NBMs), i.e. nanoparticles with medical applications. The working hypothesis is that, since 3D hepatic spheroid models are a better predictor of NBM safety than conventional 2D monocultures, this model could successfully bridge the gap between existing *in vitro* screening methodologies and *in vivo* animal models for assessing the toxicity effects of NBMs.

Two NBMs are used for the proof-of-concept study: (i) LipImage™815 and (ii) NR668 PEBCA, two dye-loaded NBMs with applications in drug delivery and deep tissue imaging. To date, these NBMs have never been tested in hepatic mono- and multi-cellular hepatic spheroids. 20nm gold nanoparticles (AuNPs) are also used as benchmark NBM, as their toxicity was previously assessed in HepG2 monoculture spheroids. 3D spheroids were formed in scaffold-free, non-adherent environments, and their liver-specific structure and function assessed using a variety of techniques, e.g., microscopy, histology, and ELISA. NBM characterisation was undertaken before 2D and 3D cultures were treated with each NBM and assessed using epifluorescent and confocal microscopy to determine uptake profiles of NBMs in both culture types. Finally, quantitative *in vitro* safety assessment was assessed in 3D spheroids using a variety of commonly used assays for NBM pre-clinical assessment, i.e., cytotoxicity and genotoxicity.

Results indicate that 3D hepatic mono- and multi-cellular spheroids display *in vivo*-like cellular morphology, secondary structure formation, expression of localised hepatic

transporters, and enhanced liver-specific functions when compared to 2D cultures. Furthermore, NBMs accumulate in 3D spheroids in a pattern similar to what is observed in *in vivo* studies reported in the scientific literature. 2D cultures are more sensitive to the effects of NBMs, with 3D monoculture HepG2 spheroids exhibiting greater resistance, in a similar manner to what is observed *in vivo*. When incorporating an NPC cell line into the model (HepG2/LX-2 multicellular spheroid model), greater variation and less resistance to NBMs is observed.

Overall, this work shows that hepatic spheroid models are promising advanced *in vitro* models for toxicity assessment of NBMs and may also help with bringing forward the 3Rs (Replacement, Reduction and Refinement) principle in animal research. Future work will include probing the expression of various enzymes involved in drug and NBM metabolism, i.e., cytochrome P450 (CYP450) enzymes, within the 3D hepatic spheroids developed. As mitochondrial dysfunction and damage has implications in up to 50% of hepatotoxins and many NBMs, it may be of interest to assess mitochondrial functionality in spheroids and apply this to toxicity screening of NBMs. A detailed study of how each NBM is internalized into spheroids, i.e., via active or passive transport, may also be of interest. As multicompartmental microfluidics devices are now commercially available for many sources, it would also be useful to extend the model into an organ-on-a-chip model.

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Abbreviations

ADME: Absorption distribution metabolism elimination

ASGP: Asialoglycoprotein

AST: Induced Aspartate aminotransferase

ATP: Adenosine triphosphate

AgNP: Silver nanoparticles

APAP: Acetaminophen

aPTT: Thromboplastin time

AuNP: Gold nanoparticle

CAGR: Compound annual growth rate

CYP: Cytochrome P450

CV: Coefficient of Variation

DAMPs: Damage-associated molecular pattern

DILI: Drug Induced liver injury

DLS: Dynamic light scattering

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DSB: Double strand breaks

EC: European Commission

ECM: Extracellular matrix

ELISA: Enzyme-linked immunosorbent assay

EMA: European Medicines Agency

EPR: Enhanced permeability and retention

EU: Endotoxin units

EUNCL: European Union Nanoparticle Characterization Laboratory

FDA: Food and Drug Administration

FIAU: Fialuridine

FOLFIRINOX: Fluorouracil (5-FU), leucovorin, irinotecan and oxaliplatin

HDL: High density lipoprotein

HCC: Human hepatocellular carcinoma

HCl: Hydrochloric acid

HCS: High content screening

hiPSCs: Human-induced pluripotent stem cell

HPRA: Health Products Regulatory Authority

HSC: Hepatic stellate cell
 HTP: High throughput
 ICP-MS: Inductively coupled plasma mass spectrometry
 IONP: Iron oxide nanoparticles
 ISO: International Organization for Standardization
 KC: Kupffer cells
 kDa: kiloDaltons
 LAL: Limulus amoebocyte lysate
 LDL: Low density lipoprotein
 LDH: Lactate dehydrogenase
 LPS: Lipopolysaccharide
 LSEC: Liver sinusoidal endothelial cells
 MAA: Marketing authorisation application
 MCTS: multi-cellular tumour spheroids
 MNP: Magnetic nanoparticle
 MPS: mononuclear phagocyte system
 MRI: Magnetic resonance imaging
 MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
 MultiFun: Multifunctional nanotechnology for selective detection and Treatment of cancer
 NAFLD: Non-alcoholic fatty liver disease
 NASH: Non-alcoholic steatohepatitis
 NDA: Non-disclosure agreement
 NK: Natural killer
 NCI: National Cancer Institute
 NoCanTher: Nanomedicine upscaling for early clinical phases of multimodal cancer therapy
 NPC: Non-parenchymal cells
 NSCLC: Non-small cell lung cancer
 NTA: Nanoparticle tracking analysis
 OECD: Organization for Economic Cooperation and Development
 PBS: Phosphate buffered saline
 PCC: Physicochemical characterization
 PCTS: Precision-cut tissue slices
 PEG: Polyethylene glycol
 PHH: Primary human hepatocyte
 PLGA: poly(lactic-co-glycolic acid)

PT: Prothrombin time

RES: reticuloendothelial system

ROS: Reactive oxygen species

siRNA: Small interfering ribonucleic acid

SPION: Superparamagnetic iron oxide nanoparticles

SSB: Single strand breaks

SWCNT: Single walled carbon nanotubes

TEM: Transmission electron microscopy

ULA: Ultra low adherence

USNCL: United States Nanoparticle Characterization Laboratory

WHO: World Health Organization

2D: Two dimensional

3D: Three dimensional

Chapter 1

Introduction

Chapter 1 - Introduction

1.1 The birth of nanotechnology and the importance of nanomaterials in medicine

Nanomaterials are used in several fields including chemistry, electronics, bioengineering, medicine and biotechnology due to their remarkable magnetic, biological, optical, electrical, catalytic, and mechanical properties, which originate from their finely tuned nanostructures and nanoarchitectures [1]. As detailed by EC recommendations, the accepted definition for nanomaterials is ‘any natural, incidental or manufactured material which comprises of particles, either unbound or in an aggregated state, where one or more external dimensions has a size range of between 1 – 100 nm for $\geq 50\%$ of the particles, according to size distribution’ [2] (Figure 1.1).

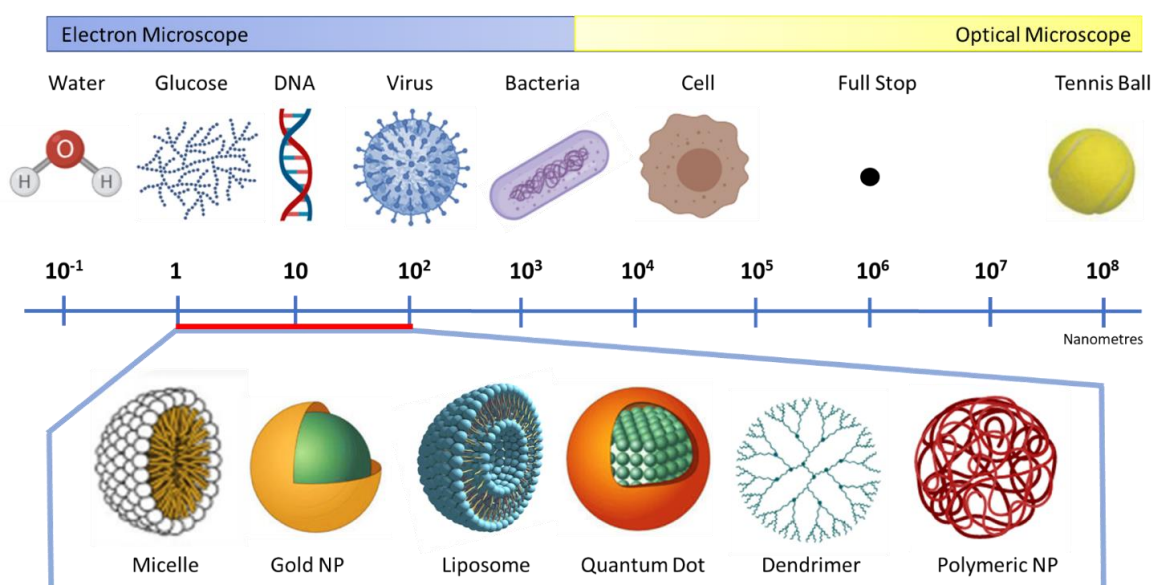


Figure 1.1. Size comparison of nano-biomaterials (NBMs) to common molecules and objects.

Nanomaterials are described as natural, incidental and manufactured materials, ranging from between 1 – 100 nm in size. Figure modified from [3] using Biorender.

Owing to its wide variety of uses, the global nanotechnology market is expected to grow in the coming years, with a compound annual growth rate (CAGR) of around 17% during the forecasted period of 2018-2024 [4]. In 2017, the global nanotechnology market accounted to nearly \$49 billion, with a projected increase to \$76 billion by the end of 2020 [5]. As detailed in the ‘Global Nanotechnology Market Outlook 2024’, the impressive growth of this field is owing to various factors including an increase in both private sector and

government function for R&D, partnerships between different countries, and an increased demand for more affordable, efficacious and smaller devices and materials. To date, the healthcare field that is largest sector where nanotechnology has made major breakthroughs. The focus of this Ph.D. thesis is in the application of nanotechnology in medicine, i.e. nanomedicine, with specific focus on and the hepatotoxicity of nanobiomaterials (NBMs) that have application in the treatment and diagnosis of cancer. Toxicity is a broad term, with its definition varying depending on the area one is discussing, however with regard to the work presented in this thesis, toxicity refers to the ability of NBMs to negatively impact cellular and genetic (DNA) health. The term hepatotoxicity specifically refers to the ability of these materials to specifically damage the liver, i.e. hepato-cytotoxicity and hepato-genotoxicity. Nanomedicine is defined by the European Science Foundation as the use of nanomaterials for diagnosing, controlling, monitoring, preventing and treating diseases [6, 7]. The nanomedicine market as a whole had a staggering estimated value of approximately \$53 billion in 2009, and it is estimated to achieve a total market value of up to \$334 billion by 2025 [8]. This large valuation will increase in the coming years due to the fact many first-generation nano-based therapies will soon go off-patent, coupled with an increasing number of new generation nano-based therapies entering the market (an approximate 40% of nanomedicines are currently in Phase 2 clinical trials), creating even more market value [9].

1.2 Clinically approved nanomedicines

There are currently several nanobiomaterials (NBMs), i.e. nanoparticles designed for biomedical applications, that have been approved by the Federal Drug Administration (FDA) and the European Medicines Agency (EMA) for their use in the clinic as nanomedicines, either therapeutics or imaging agents. The primary types of nanomedicines approved to date are either lipid-, polymer- or protein-based, with lipid-based nanomaterials being the most common. There are currently 14 lipidic NBMs which are clinically approved for varying indications, with over 20 currently enrolled in clinical trials [10]. Below I describe some of the main nanomedicines currently approved for clinical use and their application.

1.2.1 Therapeutic Nanomedicine

One of the most common therapeutic uses for NBMs is in the treatment of cancer. Here, NBMs are used either as chemotherapy carriers, or their physical properties are harnessed for other uses such as magnetic hyperthermia or deep tissue imaging [11]. The key advantage associated with NBMs' application in oncology, is their preferential accumulation in tumour

sites via the Enhanced Permeability and Retention (EPR) effect, i.e. the mechanism by which NBMs accumulate in tissues that have a greater vascular permeability, such as cancer sites or sites of inflammation [12].

Most oncological nanomedicines are liposomal formulations with anti-cancer compounds encapsulated within the liposomes. These lipid-based NBMs have many properties that make them ideal candidates as drug delivery agents, including their large biocompatibility and ability to deliver potentially toxic drugs in a targeted manner. Other advantages of liposomes include their ability to improve the solubility of both lipophilic and amphiphilic drugs, the sustained release of encapsulated material for systemic and local administration, their mechanisms of passive targeting to immune cells, their mechanisms of either targeting or avoiding certain tissue sites, and their improved tissue penetration [13]. The first clinically approved liposome oncological therapy was Doxil[®], a polyethylene glycol (PEG) functionalized liposome encapsulating doxorubicin, which can evade the reticuloendothelial system (RES) and can remain in circulation for prolonged periods with extended drug half-life. The encapsulated doxorubicin has a high and stable remote loading, driven by the transmembrane ammonium sulphate gradient, allowing for release when drug reaches tumour site [14]. Doxil[®] is currently used in the treatment of multiple cancers, including multiple myeloma, breast and ovarian cancer and Kaposi's sarcoma. Following the approval of Doxil[®], other liposomal formulations have been approved by the FDA, including Marqibo[®] [15], (liposomal vincristine), used to treat various cancers including acute lymphocytic leukaemia, acute myeloid leukaemia, Hodgkin's disease, neuroblastoma, and small cell lung cancer among others, and DaunoXome[®] (liposomal daunorubicin), used to treat a variety of leukaemia's and Kaposi's sarcoma [16]. More recently Onivyde[®], a liposome encapsulating irinotecan which has been approved by both the FDA and EMA and has shown positive indications for the treatment of pancreatic cancer. Other non-PEGylated liposomes such as Myocet[®] (liposomal doxorubicin), used in combination with cyclophosphamide to treat metastatic breast cancer, and MEPACT[®] (liposomal mifamurtide), used to treat osteosarcomas in children, teenagers and young adults, are approved for use in Europe by the EMA [17]. With the exception of Doxil[®] and Onivyde[®], all liposomal nanoparticle formulations are non-PEGylated, a surprising fact considering the many advantages associated with the PEGylation of NBM surface [18, 19]. However non-pegylated liposomes, such as Myocet[®], do have a better safety profile when compared to PEGylated liposomes, as they avoid the dose-limiting and cumulative toxicities associated with PEGylation, e.g. hand-foot syndrome (HFS), a condition which not only causes significant discomfort but also interrupts treatment regimens [20].

To date, Abraxane[®], a 130 nm albumin-bound paclitaxel formulation used in the treatment of several malignancies including metastatic breast cancer and non-small cell lung cancer (NSCLC), is the only non-liposomal approved formulation clinically approved [21]. Abraxane[®], an anti-microtubule agent that promotes assembly of microtubules from tubulin dimers and stabilising microtubules by preventing depolymerization, is advantageous due to its solvent-free formulation, which negates some of the side effects and toxicities observed with conventional solvent-based taxanes [22].

Superparamagnetic iron oxide nanoparticles (SPIONs), which I have discussed in detail in a book chapter published in 2018 (Chapter 14; ISBN 9780367901936) [23], are also clinically useful and have made significant advancements in the area of iron replacement for anaemic patients [24, 25]. Whilst parenteral iron has been used for decades as iron oxyhydroxide complex, the issue of severe toxicity stemming from injection of free iron was a major caveat [26]. This problem was circumvented by the development and introduction of nanoparticles consisting of an iron core, surrounded by carbohydrate shells consisting of various molecules such as dextrin, sucrose, dextran or gluconate [25], with the overall therapeutic goal being to increase the iron concentration in the body. Despite the advantages associated with NBMs as iron-replacement therapies, it is worth noting that of all the FDA approved formulations, very few remain active in clinical trials. Examples of this include DexIron[®], Diafer[®], Ferrisat[®], and Ferrlecit[®], which however are showing very limited activity. In contrast, Ferumoxytol[®] has shown great promise as an iron replacement therapy, but in more recent years additional approval has been sought for its applications in the field of imaging [27].

All clinically approved formulations are passively targeted, with no active or chemical-based targeting moieties, despite the widely detailed advantages of active-targeting strategies proven in the preclinic setting [28, 29]. However, at clinical level, targeted NBMs have shown major limitations, including non-specificity of the targeting ligand causing accelerated clearance from the blood, immunogenicity, impaired tumour penetration in comparison to non-targeting NBMs, and dose-reduction due to receptor-mediated endocytosis and lysosomal digestion [30].

1.2.2 Diagnostic nanomedicine

With regard to diagnostics, NBMs such as SPIONs and dye-loaded liposomes, i.e. spherical-shaped vesicles formed from phospholipid bilayers, can be used as contrast agents for MRI or in deep tissue imaging [31-33]. One such example of this is LipImageTM815, a novel 50nm IR780 dye loaded lipidic nanoparticle, useful for long term and sensitive *in vivo* deep tissue

imaging, in the near infrared region [33], and one of the NBMs which has been studied within this Ph.D. project. The enhanced optical properties associated with LipImage™815, coupled with its prolonged circulation and limited toxicity, make it ideal for *in vivo* applications. Its enhanced brightness and the very long signal duration of the encapsulated IR780 make it a safe, sensitive and long-term option for deep tissue fluorescent imaging [34]. However, many NBMs for imaging have been discontinued in both the EU and USA due to issues with regulation and marketing [35], and many EMA and FDA approved contrast agents show very low activity in current clinical trials due to a series of issues including nonspecific biodistribution, fast clearance, and short blood half-life [36].

1.2.3 Other applications of nanomedicine

Liposomal formulations have been approved for other clinical applications. Examples of these applications include Diprivan® (Propofol) [37, 38], approved by the FDA in 1989 as a general anaesthetic, Inflexal®V, a vaccine for hepatitis A [39] and Epaxal®, an influenza vaccine; however, in more recent years both these vaccines have been phased out from clinical use. The most recent and exciting advancement in the area of liposome-based vaccines is in the field of COVID-19 prevention, where two formulations have been developed and one has recently achieved approval by both the FDA and EMA. Both vaccines (BNT162b2, developed by the small German start-up BioNTech in collaboration with Pfizer, and Moderna's mRNA-1273) are mRNA vaccines delivered via liposome. If successful, mRNA-1273 and BNT162b2 would not only add to the slowly growing list of nanomedicines approved for human use but will also assist in solving an unprecedented global health crisis, further demonstrating the impact nanomedicine can have on a global scale.

Liposomal formulations have also been used to treat parasitic and fungal infections. Amphotericin B, a fungicidal treatment used for invasive and systemic fungal infections, is often the best treatment choice for immunocompromised patients, however despite the minimal risk of resistance associated with it, its success is limited due to its high acute and chronic toxicity [40]. To counteract this toxicity, amphotericin B has in recent years been encapsulated in a liposomal formulation, AmBisome®, something which has greatly reduced its associated toxicities and has allowed much higher doses to be administered to patients [41]. Other nano-formulations of amphotericin B also exist and are FDA approved, and whilst not true liposomes, they are lipid-based, e.g., Amphotec® and Abelcet® [42]. Nanomedicine products also played roles in the treatment of retinal diseases, with a

liposomal form of verteporin, Visudyne[®], a semi-synthetic porphyrin derived from protoporphyrin, used to treat macular degeneration. Visudyne[®], a light-activated liposome, offers enhanced uptake of the drug in proliferative cells. Here, following direct laser light excitation, abnormal choroidal blood vessels are damaged, preventing any further disease progression and assisting in preservation of vision [43].

1.3 Safety considerations of nanomedicine products

When developing nanomedicine products, it is vitally important to evaluate their safety, ensuring that both biocompatibility and efficacy are achieved. 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. Nevertheless, when considering nanomedicine toxicity, it is safe to say that some NBM classes exhibit much greater cytotoxic and immunotoxic potentials than others. Many studies have demonstrated that NBM safety and biocompatibility is highly dependent in fact on their physicochemical characteristics, i.e. size, charge, surface characteristics, morphology. Described below is a short summary of how NBMs can elicit toxicity in humans.

1.3.1 Mechanisms of NBMs induced toxicity

The mechanisms by which NBMs induce adverse responses in the human body can be determined on several levels - organ, tissue, cellular and molecular.

The organs most affected are those which accumulate high levels of NBMs, such as the liver (the organ of interest for this thesis), kidney, lung and spleen following IV injection, or the lung following inhalation [44-46]. Various *in vivo* studies have demonstrated how several NBMs accumulate in the liver triggering extensive toxic effects. Liver dysfunction induced by NBMs leads to structural changes in the liver, including increase in organ weight, which in turn leads to changes in liver coefficients [47]. For example, with regard to SPIONs, the mechanism of their toxicity arises from their enhanced accumulation in the liver, leading to a local immune response that can further lead to focal necrosis. One of the key mechanisms for this is that iron, both as a reactant and a catalyst, can trigger free radical production, which in turn promotes oxidation of proteins, lipid peroxidation and DNA damage [48]. Silver nanoparticles (AgNPs) have also been shown to induce severe hepatic necrosis and haemorrhage, as well as multifocal peribiliary microhaemorrhages, which affects the liver further. Severe bleeding around the biliary tract and portal vein endothelial injury has also

been noted in mice treated with AgNPs [48, 49]. In the livers of C57BL mice, gold nanorods have been shown to pre-activate liver macrophages, significantly aggravating liver damage and disease activity in mice who have immune-mediated hepatitis [50]. Titania oxide (TiO₂) nanoparticles have also been shown to induce alterations in liver structure, including increased density of liver tissue collagen, fibrosis initiation and thickening of Glissons capsule [51].

With regard to NBM impact on tissues and focusing on the liver as the main interest of this project, TiO₂ nanoparticles have been shown to cause pathological changes in liver tissue, including increase of fibrosis, hepatocyte necrosis, histiocyte proliferation and vasodilation, with these injury effects persisting and becoming more obvious one and three months post infusion [51]. They have also been shown to cause central venous congestion and hepatic sinus dilatation [52]. Silver NBMs have been shown to cause significant hepatocyte necrosis, which has been shown to cause bleeding around the biliary tract, including the gallbladder wall, as well as portal vein endothelial injury [49]. AgNPs also induce damage via inflammation and inhibiting fatty acid oxidation. They are also shown to promote the transformation of NAFLD to steatohepatitis [53]. It has also been found that gold nanoparticles (AuNPs), specifically gold nanorods, induce pre-activation of hepatic macrophages, which significantly aggravate liver injury and disease activity in mice who have immune-mediate hepatitis [54].

It is perhaps on a cellular level where NBMs elicits their most dramatic effects. One of the major mechanisms of NBM-induced cytotoxicity is membrane disruption and damage. After the integrity of an organelle membrane is damaged, inflammatory factors such as Interleukin-8 (Il-8), Interleukin-6 (Il-6), Interleukin-1 (Il-1) and Tumour necrosis factor-alpha (TNF- α) are secreted by cells, and the contents of the organelle can leak out, causing cell stress, which in turn interferes with both organelle and cell function. Several NBM formulations have been shown to trigger lysosomal membrane dysfunction, including polymeric and metal-based nanoparticles [55-57]. The primary result of this is a release of iron, protons and hydrolytic enzymes within the cell cytosol, which in turn can cause reactive oxygen species (ROS) formation, endoplasmic reticulum (ER) stress, dysfunction of the mitochondrion, and protein aggregation [58]. The formation of ROS itself, which causes DNA, protein and lipid damage, is a further proposed mechanism of NBM induced toxicity. Here, NBMs induce ROS due to acellular factors such as particle surface, size composition, and presence of metals, or via cellular responses including NBM-cell interaction, immune cell activation and mitochondrial respiration, all of which are precursors to further pathological damage including inflammation, genotoxicity, and with regard to the liver,

fibrosis, i.e. thickening or scarring of liver tissue [59]. Today, ROS generation is one of the most frequently reported NBM-associated toxicity mechanism [60] (Figure 1.2). Outside of fibrosis, excessive ROS production plays a key role in sepsis-mediated liver failure [61], and various NBMs including cerium oxide (CeO_2), TiO_2 and Ag nanoparticles have been shown to promote ROS generation, inducing oxidative stress and activating apoptotic protein signalling in the liver [62].

On a molecular level, NBMs interact with biomolecules which reside in biological fluids. Due to the surface energy of the NBMs, these form a coating on the surface of the particle, known as the protein corona [63]. Comprising of more than 100 varying proteins [64], the formation of a protein corona has the potential to greatly alter the structural properties of an NBM, including its surface charge, size and shape. The protein corona can not only cause an increase in NBM size, but can also change their zeta potential, causing NBMs to aggregate [50]. In addition to this, endogenous biomolecules which are exposed to NBMs can undergo both structural and functional changes, something which has great implications for the safety of NBMs [48].

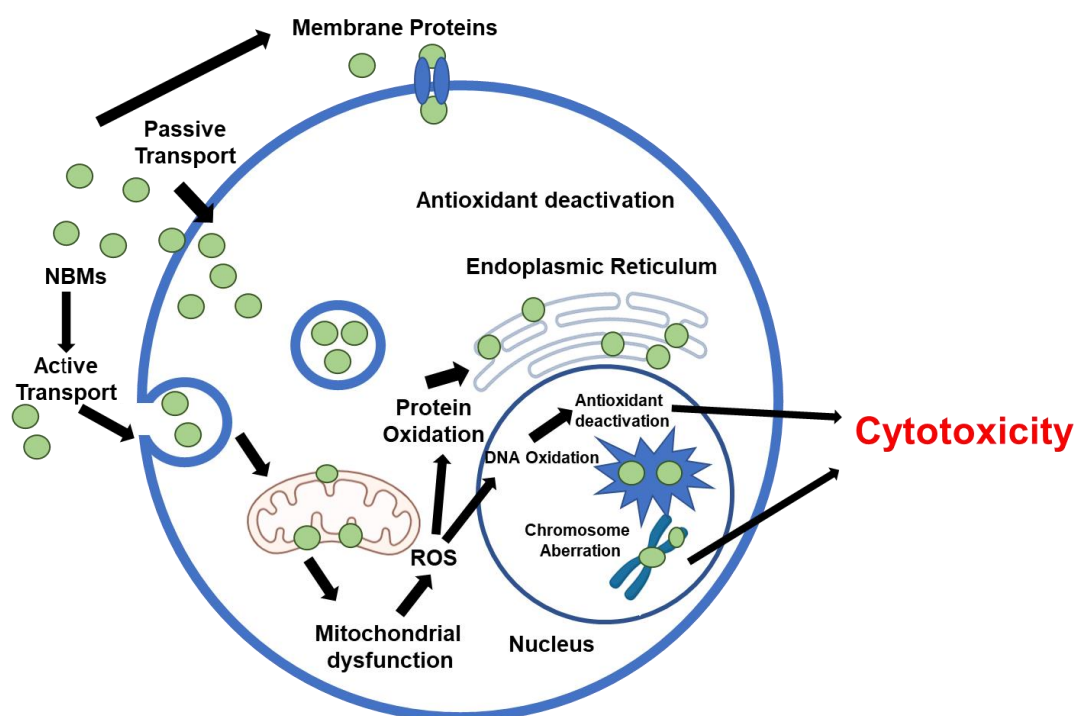


Figure 1.2. Routes of NBM entry into the cell and the mechanism of ROS-induced cytotoxicity.

Figure adapted from [65].

1.3.2 Pre-clinical assessment of NBMs

Due to their complex formulations and varying mechanisms of toxicity, a wide array of challenges is presented to researchers during the pre-clinical assessment of nanomedicines. Whilst some of these challenges are not unique to nanomedicine and are common in the development of other pharmaceutical products, it can be said that the complex characteristics of NBMs does introduce further complexities, making their translation from bench to bedside more complicated than their free-drug counterparts. Interference with commonly used *in vitro* screening strategies, is the most common problem associated with the pre-clinical testing of nanomedicines [66, 67]. When evaluating NBM toxicity, in fact, is it of vital importance to choose assessment methods and pre-clinical assays which are suitable for NBM screening. Many toxicity tests currently in use are developed for testing molecules in solution, and do not take into account the physicochemical properties of NBMs [68] and their tendency to aggregate and sediment. The optical properties of some NBMs can also interfere with readouts from some of the most commonly used cell-based *in vitro* assays that have a fluorescence output. An excellent example is the tetrazolium dye-based cytotoxicity assay (MTT), where many nanoparticles have been found to interfere with the assay's tetrazolium salt component, leading to inaccurate measurements of viability. One of the best examples of this can be seen in a paper published by Kroll *et al.*, where 24 different engineered nanoparticles were tested using four classical *in vitro* assays. The key finding from this study was that NBMs greatly interfere with classic cytotoxicity assays in a particle-, concentration- and assay-specific manner, which strongly suggests that the predictive value of each *in vitro* assay must be evaluated for every single NBM type in order to accurately assess NBM toxicity [69].

1.3.2.1 The need for a European Nanomedicine Characterization Laboratory (EUNCL) and its pre-clinical assessment assay cascade

In light of the hurdles associated with the pre-clinical screening of NBMs detailed above, the European Nanomedicine Characterization Laboratory (EUNCL) research framework was established in 2015 to harmonize and streamline the pre-clinical assessment of nanomaterials, with the overall aims of accelerating the translation of nanomedicine-based therapies to the clinic. Comprising 8 partners, 7 throughout Europe (including our collaborators at SINTEF, Norway, and CEA-Leti, France) and 1 in the US and including the Nanomedicine and Molecular Imaging Group of Trinity College Dublin, where the work

described in this thesis has been carried out, the EUNCL was funded under the Horizon 2020 programme of the European Commission and received a total of almost €5 million in budget. The unique concept of the EUNCL is the multi-competence services it provides, with each partner validated in a particular area, *i.e.*, either the physical, chemical, *in vitro* and *in vivo* biological characterisation of nanomaterials for medical applications. The EUNCL has strong links to the United States Nanotechnology Characterization Laboratory (USNCL), founded in 2004 by the National Cancer Institute (NCI) to assist in the acceleration of promising nano-based therapies and diagnostics [70]. The primary objectives of the EUNCL is to reach a high level of international excellence with regard nanomedicine characterisation, with their services made available to anyone who is developing a promising candidate nanomedicine, prior to their submission of it to the appropriate regulatory agencies in order to obtain approval for in human clinical trials, and later on if successful, marketing authorization. EUNCL are also closely aligned to the EMA, in order to permanently adapt their services to the needs and requests of regulators [71]. Based on the expertise of the United States Nanomedicine Characterization Laboratory (USNCL) and organized and operated according to the highest EU quality and regulatory standards, the EUNCL have developed their cascade of 42 validated and standardised assays to cover all areas of the pre-clinical assessment of promising nanomaterials, ranging from physicochemical characterisation (PCC) to *in vitro* laboratory safety and *in vivo* animal studies, detailed in Figure 1.3. An SOP developed for the assessing NBM-induced DNA damage, *i.e.* genotoxicity, an area often overlooked in the pre-clinical assessment of NBMs, has been developed as part of this Ph.D. project and has subsequently been validated by two collaborating laboratories and accepted as an EUNCL protocol (See Chapter 4).

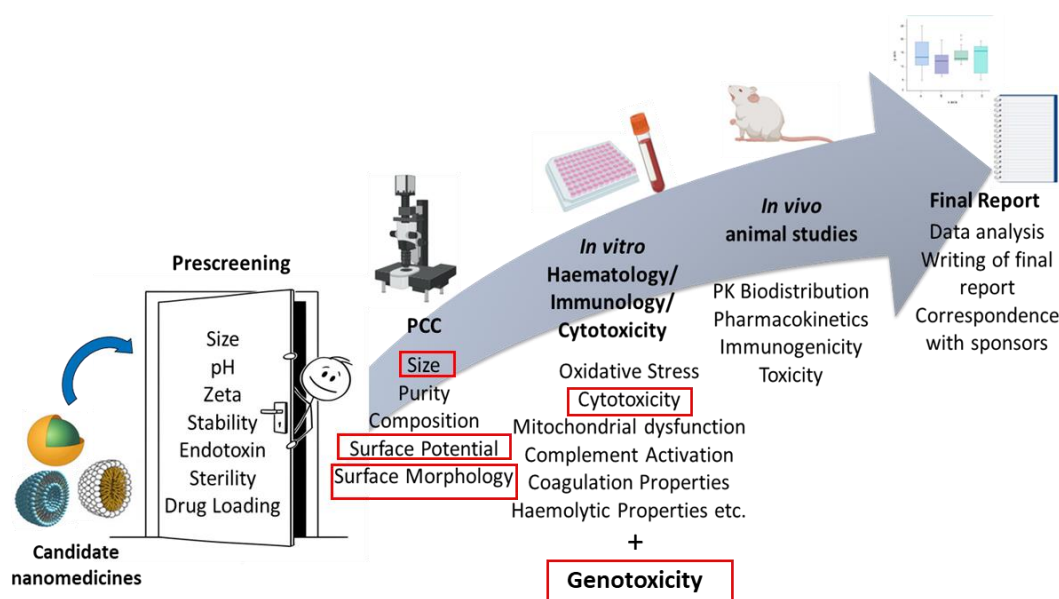


Figure 1.3. The EUNCL pre-clinical assay cascade.

Following a pre-screening step, physicochemical characterization (PCC) is undertaken on NBMs, followed by the in vitro assessment of their interactions with blood components, the immune system and cells. Once the NBM is shown to be safe to inject into animals, in vivo studies are undertaken. Red boxes denote the parameters assessed as part of my Ph.D. project, including the development of an SOP for assessing NBM genotoxicity, an area largely ignored in the current pre-clinical assay cascade of NBMs. Figure adapted from EUNCL website (<http://www.euncl.eu/>)

The development of nanomedicines for biomedical applications is an extremely broad and complex area, and due to the varying complexities of nanomaterials, the extensive characterization of these materials is often troublesome for researchers. Responding to the needs of both regulators and scientists, the EUNCL provide an invaluable service for assisting in the acceleration of NBMs to the clinic.

1.3.2.2 The Horizon 2020 REFINE project

Over the last decade, the Nanomedicine and Molecular Imaging Group at Trinity College Dublin – led by Professors Yuri Volkov and Adriele Prina Mello- have been involved in multiple large-scale multinational collaborations, aiming to assist translate NBMs to the clinic for applications in both medical imaging and cancer therapy. These collaborations bring together experts from a variety of different areas and they have been hugely beneficial in advancing knowledge in the area of nanomedicine translation. As there is the need to assess the acute and chronic impact of exposed NBMs, considerable effort has been invested in developing technically sound and industrially acceptable methodologies for screening these materials. These tasks have been taken on within a large European Commission Horizon 2020 project among several partners. The REFINE project (grant agreement ID: 761104), which started in December 2017 and is due to end in November 2021, has a budget of €8 million and also in part funded my Ph.D. project. REFINE proposes a regulatory science framework for the risk assessment of NBMs that could be used for medical technology applications, and studies/predicts the physiological distribution of NBMs, as well as developing and validating various new analytical and experimental methods and assays which are requested by regulators. The experimental methods and assays designed are stringently tested for their quality and robustness in open collaboration between partners,

ensuring the highest level of standardization possible. A significant area of interest for the REFINE project, and the focus of this thesis, has been the introduction of hepatic spheroids into the pre-clinical assessment cascade for NBMs. Throughout my project I have assessed the suitability of 3D hepatic spheroid models to predict NBM off-target effects and toxicity and have attempted to determine whether they have a higher predictive value when compared to their 2D *in vitro* cell culture counterparts. The rationale behind this work was to establish protocols and assays which could be successfully applied to the assessment of NBM hepatotoxicity specifically in 3D spheroids cultures, and in the case of the alkaline comet assay, revising existing OECD guidelines for use specifically with NBMs (See Chapter 4). By undertaking this work, it was possible to establish a validated safety workflow screening NBMs that have applications in various areas of medical technology.

1.3.3. Pitfalls associated with current pre-clinical assessment methods when screening hepatotoxicity

In addition to the challenges described previously for the preclinical screening of NBMs, a considerable challenge for researchers today is accurately predicting human-specific liver toxicity prior to clinical trials [72]. Adverse drug reactions have emerged as the primary cause of withdrawal of medications from the market, with hepatic toxicity the largest root cause for withdrawals (and black box warnings) [73, 74]. Hepatotoxicity is not only the most cited reasons for withdrawal of approved drugs, but also a primary cause of attrition in pre-launch drug candidates [74]. 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 - Questions have been raised on the relevance of animal models in pre-clinical assessment [75, 76], and over the ability of conventional, simplified *in vitro* cell based assays to adequately assess new drug candidates/NBMs [77]. In retrospective analysis of marketed pharmaceuticals, basic cytotoxicity assessment methods undertaken using hepatic cell lines have in fact been shown to fall short as predictors of toxicity [78]. This issue also affects the screening of nanomedicine products, hindering the clinical translation of NBMs [79, 80]. Therefore, accurate strategies to detect and minimize hepatotoxicity early on in the pre-clinical assessment is vitally important.

1.4 Human hepatic physiology and the importance of the liver regarding NBMs

From production of bile to metabolising a large array of compounds, including NBMs, the liver plays many important roles in the human body. As an organ, the liver is the largest in humans, and has a diverse and varied cellular composition containing hepatocytes, parenchymal cells which comprise much of the liver at approximately 80 % of total liver mass, and non-parenchymal cells (20% liver mass) in the form of Kupffer cells (KCs - liver resident macrophages), liver sinusoidal endothelial cells (LSECs – specialized endothelial cells), hepatic stellate cells (HSCs – pericytes which are the main effectors in fibrosis), fibroblasts, biliary epithelial cells, various other immune cells and adult stem cells [81].

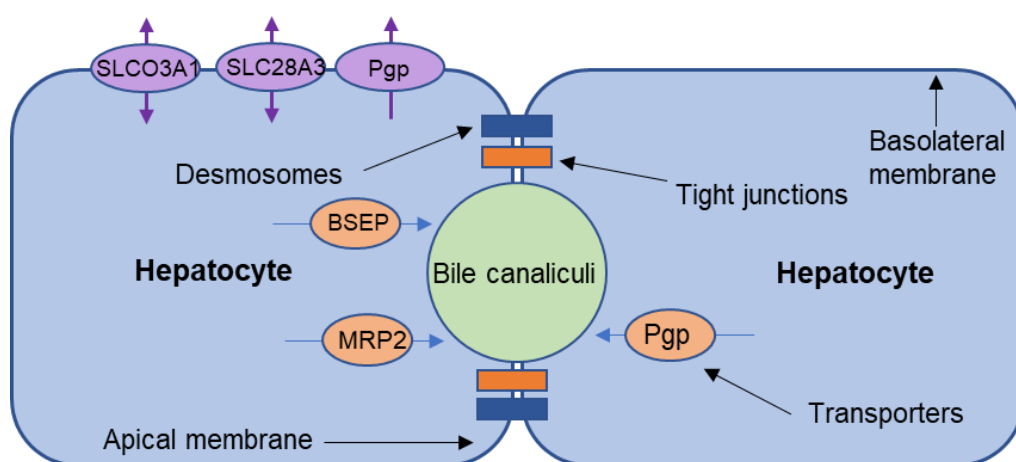


Figure 1.4. Hepatocyte zonation.

Tight junctions and desmosomes are visible on apical membrane between adjacent hepatocytes. Membrane of each hepatocyte changes to form a bile canaliculi structure. In order to facilitate efficient transport, transporters are localised to specific membranes, e.g., MRP1 is localised to the basolateral membrane and MRP2, Pgp and BSEP are seen on the canalicular membrane [82, 83].

Divided into four lobes of unequal size and shape, the liver is connected to the hepatic artery carrying blood from the aorta, and the portal vein which carries blood from the gastrointestinal tract (GIT), spleen and pancreas. Each liver lobe is complex, composed of up to one million lobules, which are hexagonal in shape and contain a central vein in their core. This macroscopic structure leads to characteristic hepatic zonation in each lobule, containing a periportal zone (zone 1) situated closer to vasculature and in contact with highly

oxygenated blood and nutrients, an intermediary zone joining the periportal and perivenous zones, and the perivenous zone nearest the central vein which has a lower nutrient and oxygen supply (known as zone 3) [84, 85]. This formation leads to functional zonation (Figure 1.4), where hepatocytes in varying lobule zones exhibit different hepatic functions. Various metabolic process, including glycolysis, synthesis of amino acids, production of bile acids, glucose uptake and glucuronidation are all favoured in the perivenous cells, which also show enhanced cytochrome P450 (CYP450) activity. In contrast to this, the periportal cells show comparatively greater oxygen uptake, urea synthesis, oxidation of fatty acids, synthesis of cholesterol, sulfation and gluconeogenesis. NPCs such as stellate cells, Kupffer cells and bile duct cells are also more abundant in the periportal zone [86]. For more specific details and comprehensive reviews of the general anatomy and function of the liver please refer to Abdel-Misihs and Bloomstons 'Liver Anatomy' [81] and Bacon *et al.* 'Comprehensive Clinical Hepatology' [85].

The liver is also a key organ of interest for NBM toxicity responses, for a variety of different reasons. [87, 88]. Approximately 30 – 99 % of NBMs accumulated and are sequestered in the liver following administration, reducing the amount of NBMs reaching the target tissue and potentially leading to unintended hepatotoxicity. Thus, the interactions between NBMs and liver cells determines the fate of the NBM *in vivo*; however, to date, the specific combinations of physicochemical properties that determine sequestering to the liver remains unknown. Whilst *in vivo* studies mainly focus on NBM accumulation at organ level, *in vitro* assessment focuses on a single hepatic cell type in culture, not considering the unique 3D arrangement and architecture, as well as cellular composition, of the liver and how these affect NBM interactions. For example, it is widely detailed in literature that many NBMs are taken up by NPCs despite these being the less abundant cell type in the organ, and that NBMs which are taken up by hepatocytes are largely cleared in the body by the hepatobiliary pathway. The interaction between NBMs and various specific liver cell types is discussed further below.

1.4.1 Role of hepatocytes in the liver

The primary functioning cell type in the liver is the hepatocytes, cuboidal hepatic epithelial cells which line the sinusoids. They sit in plates along the hepatic lobule, between systems of capillary sinusoids that connect the portal tracts to the central vein. The central vein facilitates a consistent supply of blood and other materials. Hepatocytes are tightly connected with each other to form cell plates via junctions, including gap, tight and adherens junctions.

Gap and tight junctions play a critical role in bile secretion, one of the most differentiated functions of the liver. Hepatocytes form canalicular-like structures, which run perpendicular to capillaries and are a key component of the liver's structure and functionality [89]. Hepatocytes are also functionally polarised cells, which contain specific transporters localised to both the apical (or canalicular) or basolateral (sinusoidal) membranes. This polarity is vitally important for efficient liver functions, and it allows molecules/compounds to be taken up or effluxed into the bile, or metabolized and transported back into the bloodstream [84, 85, 89]. Hepatocytes play many important roles with regard to metabolic function, production of bile, detoxification of materials and protein synthesis [90]. They also express a variety of plasma proteins, including protease inhibitors, transporters, inflammatory modulators and albumin, and activate innate immunity as a defence mechanism against invading microorganisms by secreting innate immunity proteins [91, 92]. Regarding their interactions with NBMs, hepatocytes themselves endocytose NBMs, releasing them back in the bloodstream or into bile, however, to date, uptake of NBMs in hepatocytes has only been observed when very large doses of NBMs are administered or in instances where macrophages have been chemically depleted [93]. Various factors appear to influence NBM uptake by hepatocytes, including PEGylation and positive surface charges [94]. Size also plays a role, with uptake into hepatocytes normally occurring for NBMs below 50 nm [95]. Hepatobiliary clearance of NBMs occurs actively, promoted by various liver transporters and facilitated by a variety of drug-metabolizing enzymes, followed by either secretion into bile duct via bile canaliculi or being filtered back into bloodstream [96]. An understanding of hepatocyte targeting is critical to removing NBMs via the hepatobiliary route, and today there are various strategies for designing NBMs to both enter and interact with hepatocytes, harnessing either (1) transcytosis via the endothelial cell lining or (2) the sinusoidal intercellular junctions.

As hepatocytes make up such a vast proportion of the cell of the liver, many NBMs have also been specifically designed to target these cells [97], by the active targeting of low-density lipoprotein (LDL), high density lipoprotein (HDL), asialoglycoprotein (ASGP) and glycyrrhizin/glycyrrhetic acid receptors, and the immunoglobulin A binding protein. A detailed overview of the interactions between hepatocytes and NBMs (and other hepatic cell types) can be found in a comprehensive review from Zhang *et al.* [88].

1.4.2 Role of non-parenchymal cells in the liver

Liver non-parenchymal cells, or NPCs, make up for an approximate 20% of liver volume. NPCs play many roles in the liver, primarily in maintaining hepatic structure and functionality. Liver damage significantly alters both the phenotype and function of non-parenchymal cells, significantly reducing the ability of the liver to recover appropriately. Three of the most abundant and important NPCs of the liver are sinusoidal endothelial cells (LSECs), Kupffer cells and stellate cells. Other important NPC types that also reside in the liver but are less abundant are fibroblasts, neutrophils and macrophages. Since in my Ph.D. project stellate cells were used, these are further discussed below.

Hepatic stellate cells (HSCs; lipocytes, interstitial cells, fat-storing cells, Ito cells) represent 6% of the total liver cell population [85]. They play key roles in responding to hepatic injury, with stellate cells in a healthy liver acting as a major storage unit of Vitamin A [98]. They exist in the space of perisinusoidal Disse between the sinusoidal endothelial cells of the hepatic lobule and parenchymal cells, and also store up to 80% of all retinoids in the body, as retinyl palmitate in lipid droplets in the cytoplasm [99]. They also have the ability to detect DAMPs, and can be activated following the release of cytokines via Kupffer cells, a process which greatly aids regeneration of the liver [84]. In the healthy liver, stellate cells are inactive and maintained in a quiescent state, with their primary role as Vitamin A storage units [100]. However following activation, hepatic stellate cells form scar tissue during fibrosis, a process which involves chemotaxis, chemokine secretion, contraction and proliferation of cells.

With regard to their interactions with NBMs, NPCs play vital roles, with NBMs interacting with varying NPCs, before reaching hepatocytes.

Kupffer cells recognize NBMs as foreign materials, with NBMs being internalised through scavenger receptors and subsequently taken up by micropinocytosis, clathrin-mediated and caveolin-mediated endocytosis, and various other endocytosis pathways [88]. It is the Kupffer cells, along with the blood-circulating monocytes and macrophages in other tissues including spleen and gut, which constitute the mononuclear phagocyte system, or MPS. The MPS, also known as the reticular endothelial system, is responsible for sequestering approximately 95% of NBMs administered [101], with the rate of uptake highly dependent on NBM characteristics such as surface chemistry, size and ligand chemistry. It is reported that larger NBMs, ranging in size from 400 nm to 600 nm, are preferentially phagocytosed by these cells, along with particles that are neutrally charged (i.e., PEG coatings). Studies have also shown that rod-shaped NBMs exhibit a reduced clearance compared to their spherical

counterparts, potentially due to the presence of fewer accessible binding areas for interactions with macrophages [102, 103]. Various studies have highlighted this phenomenon, with Lunov *et al.* showing that 20 nm and 60 nm SPIONs accumulated irrespective of their size in macrophages via clathrin-mediated and scavenger receptor A endocytosis, with the larger SPIONs exhibiting up to 60 times greater uptake than the smaller ones [104]. Kupffer cells have also been shown to take up low-density lipoprotein (LDL) NBMs and liposomes of sizes ranging from 27 nm to 590 nm [105-107].

Despite their low abundance and challenging location, i.e., their residence in the space of Disse adjacent to endothelial cells, hepatic stellate cells (HSCs) have also been shown to take up various NBMs, including liposomes, AuNPs, SPIONS and polymeric NBMs [108-115]. HSC-targeting NBMs also have implications in the treatment of liver fibrosis, with surface receptors allowing NBMs to directly target HSCs, provided they have not been removed from circulation by LSECs or Kupffer cells [88].

LSECs, the cell type which forms continuous linings along the vasculature of the liver sinusoid, may also have implications for NBMs, with studies from Kamps *et al.*, Akhter *et al.*, and Kren *et al.* illustrating that they can successfully take up both AuNPs, micelles and liposomes [107, 116, 117].

1.5 *In vitro* models of the liver

Due to the central role the liver plays in the metabolism, clearance and biotransformation of drugs/NBMs, a considerable challenge for researchers today is the predictions of their human-specific liver toxicity [72]. Today, drug-induced liver injury is the most cited explanation for the removal of already approved drugs from market [118]. 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 drugs and 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 [119, 120]. For decades, *in vitro* 2D cultures of the liver have been the gold standard for determining acute hepatotoxicity of drugs and 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. The latter include for example the Huh7, C3A and HepG2 cell lines. Whilst useful early

predictors of toxicity, these immortalized cell lines have reduced metabolic competencies in comparison to their primary hepatocyte counterparts.

Furthermore, conventional 2D cells culture relies on the adherence of dissociated cells to flat surfaces, typically sterile polystyrene or glass. 2D *in vitro* models are grown in two dimensions have access to consistent amounts of nutrients and growth factors from culture medium, thus allowing for homogenous cell growth and proliferation [121]. This coupled with the low cost, efficiency, ease of use of the 2D *in vitro* culturing methods makes 2D models a very attractive pre-clinical tool. Despite these advantages, major flaws can be seen with conventional 2D culture. The vast majority of these methods provide no control over cell morphology, a factor which determines the biophysical cues which affect cell activity *in vivo* [121]. As a means of overcoming this, in recent years more advanced culture materials have been developed including micro patterned substrates such as microwells, as a means of customising cell shape to a more *in vivo* like setting. The effect of improving these *in vitro* assays may potentially lead to more effective and rapid pre-clinical drug development. Some of the most common 2D *in vitro* models of the liver are described in more detail below.

1.5.1 Primary human hepatocytes (PHH)

One of the most physiologically relevant 2D culture methodology for hepatic modelling is the use of primary human hepatocytes (PHH). PHH, which can be isolated from human liver biopsies and cultured in 2D monolayers using adherent surfaces and culture medium, are widely regarded as the ‘gold standard’ *in vitro* model for evaluating drug biotransformation, toxicity and drug-induced liver injury (DILI) [122-124]. Whilst primary animal hepatocytes are also widely available and relatively inexpensive in comparison to their human counterparts, inter-species differences in metabolism and pharmacokinetics impact their predictive value and reduce their usefulness [125]. In contrast to this, when isolated and cultured appropriately, PHH exhibit very similar behaviour to *in vivo*, including conserved uptake and excretion functions, glucose metabolism, increased albumin secretion, integration of Phase I and II metabolic pathways, ammonia detoxification, and expression of a variety of other functional markers for up to 72 hours in culture [126]. These properties make PHH the most accurate and useful of all the available *in vitro* hepatic models currently available, [127]. Despite these attractive properties, PHH do have their limitations. Their wild-type characteristics can only be maintained in two-dimensions for a limited time due to the de-differentiation imparted by flat culture surfaces. Additionally, manipulation of PHH

can lead to changes in metabolism, decline in CYP450 enzyme activity, early senescence and slow proliferation, all of which occur after a short number of passages [122, 123]. In order to avoid these issues, primary cell harvesting needs to be undertaken regularly, at large amounts of labour and cost. A further issue surrounding the use of PHH is the issue associated with seeding density, with PHH in 2D cultures seeded at densities as little as 1% of normal physiological density. As a consequence of this, intercellular signalling is severely impaired [128, 129]. Today, the main uses of primary hepatocyte cultures is in understanding the mechanisms of liver regeneration, and in drug screening, where they are gold standards due to their phenotype and function, which are more comparable to the *in vivo* environment than other 2D *in vitro* models [124, 130].

1.5.2 Sandwich cultures of primary human hepatocytes

First used by Dunn and colleagues in 1989, sandwich-cultured hepatocytes, or SCH, refers to the culturing of primary hepatocytes between two layers of collagen in sandwich form [131-133]. Culturing PHHs in this manner has many advantages, including retained polarity between cells, with the basolateral and canalicular transporters localised in the correct manner, and with successful formation of canalicular networks [134-136]. As sandwich cultures maintain their metabolic activity and successfully form canalicular networks, they are invaluable tools for studying various areas including drug transport, drug interactions and hepatotoxicity, and they can be used in the investigation between the uptake and efflux transporters and drug metabolising enzymes [136].

1.5.3 Immortalized and transformed hepatocytes

In order to overcome the caveats of interspecies differences and de-differentiation seen with using PHH, immortalized and transformed hepatic cell lines have been developed, mainly derived from human hepatocellular carcinomas. These cell lines have been gradually modified genetically or chosen for an oncogenic phenotype [137]. Advantages associated with using immortalized cell lines over PHH include their rapid proliferation and therefore long lifespan, their resistance to de-differentiation, ease of availability and handling, improved passaging, stable phenotype, increased resistance to senescence, and removal of any donor variation, factors which make them ideal cells for long term culture and experimentation. Immortalized hepatic cell lines have been used successfully to study Hepatitis B and D infections [137], with the mechanisms of hepatitis entry being elucidated in the HepaRG cell line [127, 134, 138-140], the expression and replication of Hepatitis B

revealed in HepG2 [141], and the Hepatitis D replication cycle discovered in both Huh7 and HepG2 cells [142, 143]. They have also been used successfully to investigate steatosis [144], and they can also act as appropriate models for hepatocarcinogenesis [145]. Today, immortalized hepatocyte cell lines are most commonly used *in vitro* model for drug and NBM safety and toxicity screening [146, 147]. The most widely used and well characterised human hepatic liver cell line is HepG2, the cell type used in my Ph.D. project. HepG2 cells are an epithelial-like, non-tumorigenic cell line, derived from hepatocarcinoma [134]. Despite having relatively poor basal CYP expression levels [148], a variety of studies have demonstrated that they exhibit

expression of CYP1A2 and CYP3A4 enzymes similar to that of PHH [149]. They can transform many xenobiotics, and produce multiple hepatic plasma proteins, including transferrin, fibrinogen, and albumin [150]. HepG2 cells are widely studied to enhance their function and toxicological predictivity, and in the past they have been transfected with varying CYP enzymes to enhance enzyme levels, making them a more appropriate model for predicting liver toxicity [151]. Another hepatic cell lines which is widely used for liver toxicity screening is the HepaRG cell line. HepaRG cells are a terminally differentiated human hepatocarcinoma cells line which form both cholangiocytes-like and hepatocyte-like cells which, following incubation with DMSO, can be differentiated into mature hepatocyte or biliary like cells [134, 152]. HepaRG cells are far more metabolically competent than conventional immortalized hepatic cell lines, expressing functional transporters and secreting key hepatic materials like albumin, lactate and urea, and expressing key Phase I and II enzymes, including CYPs, with high drug metabolizing potential [152, 153]. Another common example of immortalized hepatic cells is the Huh7 cell line, established in 1982 from a well differentiated cellular carcinoma and derived from the liver tumour of a 57-year-old Japanese man. This cell line is highly susceptible to HCV infection and is widely used to model hepatoma and HCV. C3A cells, a clonal derivative of HepG2, are also widely used due to their advantages over traditional immortalized cell lines including high albumin secretion, ability to be maintained in glucose-free medium and strong contact inhibition [134, 152, 154]. They appear to be a better representation of primary human hepatocytes than HepG2; however, much more experimentation and validation needs to be undertaken in order to fully understand their abilities as liver models.

Despite the advantages that immortalized cell lines provide, there are still many shortcomings associated with them [147]. The vast majority of immortalized hepatocyte cell lines exhibit altered phenotype and genomic content [155] changes in their differentiation potential, loss of cellular polarity [155] and contact inhibition, reduced CYP450 activity

[139] and induction of inflammatory mediators [156]. The expression of Phase I and II enzymes in HepG2 cells, for example, is markedly lower than that seen in PHH, making HepG2 cells not fully representative of human hepatocyte phenotype and function and reducing their ability to detect hepatotoxins as accurately or readily as human hepatocytes. Atienzar *et al.* revealed in 2014 that, whilst HepG2 cells are sensitive to compounds that damage the liver, i.e. hepatotoxins at 80%, they only have a specificity of 40% when treated with non-hepatotoxins. It is vitally important that further work is undertaken on HepG2 cells to fully elucidate their usefulness and to be fully confident in their use as a hepatic screening model. On the other hand, despite showing enhanced liver specific structure and function, HepaRG cells have increased CYP enzyme expression only when treated with high levels of DMSO-spiked culture medium, a response [134]. Due to these caveats, in recent years different strategies for culturing immortalized hepatocytes to potentially overcome these issues have been employed, ranging from culturing them in co-culture systems with primary human hepatocytes, or culturing them in various 3D systems which have been shown to increase specific hepatic characteristics [157, 158].

1.5.4 Non-parenchymal cell/hepatocyte co-cultures

In order to increase the predictive power of *in vitro* hepatocyte cultures, non-parenchymal cells (NPCs) have been co-cultured with hepatocytes [159, 160]. Much evidence suggests that the incorporation of NPCs into *in vitro* hepatic models assists in producing a more *in vivo*-like microenvironment which not only has increased liver function but also can respond or react to inflammation or damage more accurately. One of the most widely used hepatic non-parenchymal cell type used in co-culture are Kupffer cells. Literature suggests that Kupffer cells (KCs) can influence hepatocyte function, with Yagi *et al.* detailing in 1998 that metabolic functions are greatly increased when KCs are incorporated into a hepatocyte culture model [161]. In more recent years, immune cells have been cultured with hepatocytes to form co-cultures, e.g., the human monocytic cell line THP-1 co-cultured with Huh7 cells. Here it was found that when Huh7, a common immortalized hepatocyte cell line was cultured with the monocytic cell line THP-1, an increase in both pro-inflammatory and stress-related gene expression was observed following treatment with troglitazone, a well-known hepatotoxic compound. Increase in sensitivity and drug metabolism were also observed in comparison to Huh7 culture alone [162]. Kang *et al.* also discovered that co-culturing hepatocytes with liver sinusoidal endothelial cells (LSECs) resulted in maintained and enhanced CYP enzyme activity for as long as 48 days, with liver specific markers such as

albumin and transferrin, and urea maintained for up to 37 days and 28 days respectively [163]. Co-culture with hepatic stellate cells (HSCs) and hepatocytes was also undertaken. HSCs have been shown to maintain the differentiated status of hepatocytes *in vitro*, via their soluble and particulate factors and their lipid extracts. Culturing hepatocytes with HSCs not only assisted in maintaining hepatocyte function, but also displayed many strong beneficial effects with regard to enzyme activity and morphology [164].

1.6 *In vivo* models of the liver and their failures in predicting hepatotoxicity

Outside of *in vitro* experimentation, *in vivo* liver models are a vital part of the pre-clinical assessment cascade for NBMs. The use of animals in pre-clinical research provides a basic indication of the fate of NBMs in organs, whilst also providing vital information on dosing and potential toxicities. Historically, the use of animals in the prediction of human toxicities dates to as early as the late 1930s [165], and today both the EMA and the FDA generally require preclinical assessment to be undertaken in animals prior to any new drug or therapy entering human clinical trials [166].

Currently, *in vivo* animal studies of NBMs are most often undertaken in porcine or rodent models [167], in accordance with the European Commission Directive 2010/63/EU, the EU legislation on the protection of animals for scientific purposes (which protects live non-human vertebrates and foetal mammals from the last third of their normal development) [168], and they are deemed an essential component in the translation of NBMs from bench to bedside. Nevertheless, the choice of the animal model and the understanding of its intrinsic limitations are vital elements when assessing NBM toxicity and accumulation.

Rodent models have been widely used for many reasons, including their availability, low cost, small sizes, ease of handling, and fast reproduction rate. The combination of these features provides scientists with a powerful tool for understanding the mechanisms of human disease and also assessing novel drug compounds. Mouse models are also particularly suited for investigating various aspects of cancer prevention which may be difficult to study in humans, including identifying various factors or interventions which prevent growth of early tumour lesions [169]. However, as rodents demonstrate many anatomical, physiological and immune response differences compared to humans, their applicability is limited and many rodent models are unable to fully recapitulate human conditions such as Parkinson's disease and Alzheimer's disease among others [170]. Therefore, porcine models are also often used in pre-clinical assessment. The size of the pig, coupled with similarities to humans regarding anatomy and immunogenetics, make it an ideal model for recapitulating liver-specific

diseases such as human hepatocellular carcinoma (HCC), and its co-morbidities, including cirrhosis and non-alcoholic steatohepatitis (NASH) [171]. There is a strong resemblance between the central nervous system of humans and pigs, making them ideal model organisms for studying human neurodegenerative diseases such as Alzheimer's, amyotrophic lateral sclerosis (ALS) and Parkinson's disease, in a more representative manner than models previously available [172]. Porcine models are also often used for assessing NBMs, with one study from Andrasina *et al.* used pig models to assess the accumulation and effects of liposomal doxorubicin in liver tissues by radiofrequency ablation and irreversible electroporation [173].

Another emerging *in vivo* model for NBM pre-clinical assessment is the zebrafish, a non-mammal model offering new, practical and cost-effective opportunities to help bridge the gap between *in vitro* and *in vivo* studies. The popularity of the zebrafish model for studying NBMs is supported by the development of automated, high-throughput readout technologies. This model has many characteristics that make it more attractive than conventional *in vivo* models, with low husbandry costs compared to rodents or pigs. This is a hugely attractive factor for researchers, as there is normally a huge financial burden associated with NBM *in vivo* studies. Possibly the most attractive factor associated with the use of zebrafish is the fact they can be used without any ethical approval. Early-stage zebrafish are in fact not protected by the European Commission Directive 2010/63/EU on the protection of animals for scientific purposes, based on the principle that they are unable to independently feed [174]. Younger zebrafish, i.e., zebrafish larvae, are optically transparent, a factor which enables the high-resolution imaging of various events in real time. As is observed with all *in vivo* models, the question of conservation of characteristics and biological features must be questioned prior to implementing zebrafish as a suitable model for assessing NBM. Zebrafish shares many essential physiological homologies with humans [175, 176], with various other physiological parameters and organs of relevance to the biodistribution and toxicity of NBMs widely studied, including the lymphatic system, blood components, immune and vascular systems, and the liver. Given the advantages and similarities over their rodent counterparts, zebrafish and their larvae are becoming increasingly used as models for the assessment of NBMs, with accumulation, circulation, biodistribution, stability and toxicity all studied in living zebrafish. Examples of the use of the zebrafish model for the pre-clinical assessment of NBMs include works by Vibe *et al.*, who assessed the toxicity of thioridazine encapsulated PLGA particles [177], Peng *et al.* who assessed the release of hydrophobic drugs from cyclodextrin and dextran based nanocarriers [178], and Yan *et al.*

who used zebrafish to study photothermal controlled drug delivery used mesoporous silica nanoparticles [179].

Although screening toxicity in animal models has been a main aid in ensuring material safety in humans for many years, and despite the deeply rooted assumption that animals are good predictors of human toxicity, much information exists to demonstrate the contrary [180-182], with many serious questions being currently raised as to the fitness of animals to adequately predict human responses. In recent years, the conversation has switched. It has been shown that only 60% of drugs entering clinical trials are successful in Phase I (safety) trials [183], with approximately half of these failures occur due to unanticipated human toxicity, unseen in animal models. Overall, 89% of novel drugs fail in human clinical trials. Half of these failures occur due to unanticipated human toxicity. One provocative question that has been asked is “if animal studies are truly predictive of human toxicities, why are toxicity rated drug attrition rates in human trials so high?”. Notably, the consensus among several scientists is that *in vivo* animal models are not as accurate predictors of human responses as we previously believed [184]. In a 2006 review from the European Commission it was found that of 76 different animal studies only in 28 cases (37% of the studies) could be replicated in human randomised trials, while animal results were completely contradicted in humans for 14 cases (the remaining 34 cases remained untested in humans) [185]. A further review of 221 animal studies showed that only in 50% of the cases results obtained from animal experiments agreed with those generated by human trials. Here, discordance between animals and humans was found to be caused by both experimental bias and the failure of the animal model to adequately mimic human disease state [75]. When concerned with the ability of animal models to accurately predict hepatotoxicity, the results are similarly stark. A review of 230 animal studies undertaken in 2000 showed that approximately 72% of human toxicities could be associated with animal toxicity, with strong correlation between animals and humans seen with regard to hematologic, gastrointestinal and cardiovascular toxicities. However, hypersensitive and cutaneous reactions, along with hepatotoxicity, correlated poorly with animal toxicity screens [125]. This review is not a unique report. The poor prediction of human liver toxicity by animal models has been reported also by various other studies. Many possible reasons have been proposed as to why such discrepancies are seen between animals and humans. The primary reason can be linked to the intrinsic interspecies differences regarding liver physiology and metabolic capacity of animals [182]. It is reasonable to say that animals do not accurately predict human metabolism, and although toxicity screening in animals is necessary to meet specific regulatory requirements, the interspecies differences in drug absorption, distribution,

metabolism and excretion (ADME) between animals and humans necessitate the completion of hepatic *in vitro* cell experiments using human cells. As a consequence of inter-species variation, it is very possible that drugs or materials which may be hepatotoxic in humans, but not in animal models, may be brought to the clinic. This can, and has in the past, had catastrophic outcomes for patients. One examples of this failure can be seen regarding fialuridine (1-(2-deoxy-2-fluoro-1-D-arabinofuranosyl)-5-iodouracil, or FIAU), a nucleoside analogue that was developed as a potential therapy for viral hepatitis B [186]. Despite not showing any hepatotoxic indications during pre-clinical animal studies undertaken in 1993 in many species (rat, mouse, dog and cynomolgus monkey), 7 of the 15 volunteers in the study suffered lactic acidosis and acute liver failure a few weeks into Phase I clinical trials, at doses 100 times lower than those used in the pre-clinical animal studies. 5 of these patients died, and two more only survived after receiving liver transplants [186, 187]. Over 20 years later, a retrospective study by the US National Academy of Sciences analysed all preclinical toxicity studies of fialuridine and confirmed that in all animal studies undertaken there was no indication that the drug would cause human liver failure. They did however demonstrate that a specific mice model with humanized livers could in fact recapitulate the drugs toxicity, again illustrating the importance of human relevant animal liver models [188]. The catastrophic outcomes of falsely identifying a toxic drug as “safe” has also been experienced with Serazone (a drug used to treat depression) and Rezulin (a drug used to help control blood sugar levels in Type I diabetic patients), both recalled from the market for inducing severe hepatotoxicity. Safety data compiled for these drugs suggests that while some liver function abnormalities were detected during clinical trials, many other relevant factors for their discontinuation were identified, including a lack of attention being paid to rare adverse drug reactions, and a lack of predictability of animal models [189]. Many other factors have also been cited as to why such stark discrepancies are seen between animals and humans, including the heterogeneity of patients’ populations, lifestyle, environmental factors, susceptibility, and pharmacogenetic factors that may make patients more sensitive to adverse reactions than their animal experimental counterparts. Animal studies are undertaken in very well-controlled environments, something which is non-predictive of patients who live in heterogenous conditions. Normal healthy animals used in pre-clinical assessment are also non-predictive of diseased human patients. Furthermore, it is also important to consider the lifestyle, environmental, susceptibility, and pharmacogenetic factors associated with some patients, factors which may make the patient more sensitive to adverse reactions [182].

It is now suggested that animals could be either abandoned entirely or at least less widely used and potentially replaced with other more reliable, tissue-mimetic cell-based methods or advanced methods, which will be discussed in the following section. There is growing interest in the development and implementation of new research techniques which may lead to the replacement, reduction or refinements (3Rs) of animals in scientific research, with the World Health Organization (WHO), European Pharmacopoeia, FDA and EMA, among others, recognising this need and calling for the development of these ‘alternative methodologies’ [190].

In addition to not seeing key toxicities like the examples presented above, in fact, another issue is the abandonment of potentially useful treatments, or researchers being led the wrong directions from using invalid animal models that have no concordance with human biology. This not only wastes time and money, but also greatly increases the numbers of animals used in research, and the continued investment into inappropriate animal models pulls funds away from the development of more reliable, accurate testing methods. In recent years, more evolved, human-based technologies have garnered much attention and great enthusiasm, including human organ-on-a-chip models, 3D liver bioprinting, human organs grown in labs, computation technologies, and the Human Toxome Project. The primary benefit of these methodologies in the pre-clinical setting over their animal counterparts is the fact they are based on actual human biology, therefore eliminating the guesswork required to extrapolate data from animals to humans. Many of these methods are also high-throughput, and offer whole systems biology compared to conventional *in vitro* methodologies. Whilst gaining momentum, these tests (discussed in further detail below) are still in their early stages, and need to be prioritized, with much investment and attention before they can be fully implemented. Recent advances have been made in these areas, and interestingly, regulatory agencies and governing bodies are becoming more open to this idea, albeit very slowly. Increasingly scientific organizations are recognizing the huge potential of these alternatives to animal testing [182]. Whilst paradigm shift towards more relevant models, to date financial investment still greatly favours animal experimentation [191]. Further information relating to this topic can be found in a comprehensive review from 2019 entitled ‘Limitations of Animal Studies for Predicting Toxicity in Clinical Trials’ [182] and in ‘The Flaws and Human Harms of Animal Experimentation’, published in 2015 from the Humane Society Institute for Science and Policy [191].

1.7 A shift towards 3D culture and its advantages over conventional methodologies

In the past, it was proposed that high throughput *in vitro* hepatic models such as primary human hepatocyte (PHH) suspensions, multi-well perfused bioreactors and liver slices, among others, may be an invaluable resource, enabling a shift towards more rapid and sensitive alternative drug screening methodologies [192]. Despite some of these models being around since the early 2000s, however, these methods have not improved the translation rates of new drugs. Biomarker research has also been noted as an emerging area within hepatology and assessing hepatotoxicity [193], however this method has not been any more helpful than other more conventional ones.

Table 1.1: Brief overview of the differences between 2D and 3D culture, regarding morphology, response to materials, *in vivo* likeness and other key parameters.

Property	Type of Culture	
	2D	3D
Morphology	Sheet-like, flat, stretched cells grown in monolayers	<i>In vivo</i> like shape. Cells self-assemble/aggregate to natural state
Proliferation	Can proliferate at a higher rate than <i>in vivo</i>	Proliferate at faster or slower rate depending on cell type/3D system
Protein/gene expression	Often display different expression levels to <i>in vivo</i> models	Protein/gene expression levels similar to those in <i>in vivo</i> models
Access to essential materials	Unlimited access to O ₂ , metabolites, nutrients and signalling molecules	Varied access due to 3D morphology
Cell-cell interactions	Cannot recapitulate complex cell-cell/cell-matrix interactions due to flat morphology	Appropriate interactions between cell-cell & cell-matrix environments
Multi-cellular systems	Good for studying immune response	Good for co-cultures but complicated when more than 2 cell types introduced
Sensitivity to drugs/nano materials	More sensitive; often succumb to treatment more readily due to reductionist morphology	May be more resistant to treatment, better predictors of <i>in vivo</i> drug and nanomaterial response
Exposure to medium/drugs/nanomaterials	Cells grown in 2D equally exposed to nutrient/growth factors/drugs/NBMs distributed in culture medium	Depending on spheroid size, materials may not penetrate core and reach all cells
Quality of culture	Reproducible high-performance and simple but highly reductionist	Quality depends on method, but more accurate & have longer lifespan
<i>In Vivo</i> likeness	Do not mimic natural presentation of tissues	Great likeness to <i>in vivo</i> as organs and tissues are in 3D form
Cost of maintaining culture	Cheap, all reagents/materials commercially available	More expensive (depends on type), more time consuming, batch-to-batch variation if using culture substrates, less availability
Time needed for cultures	Minutes to few hours	3-4 hours to days

In recent decades there has been a move towards the creation of alternative more biologically relevant *in vitro* models which can better mimic the *in vivo* environment [194], by creating three-dimensional (3D) cell cultures (Table 1.1). The first uses of 3D cell culture date back to the 1970s, where Hamburg and Salmon developed a culture method involving soft agar solutions [195]. Since then, interest in 3D culture has increased exponentially, with many different 3D culture methods widely used today. Based on their methods of preparation, detailed in Figure 1.6, 3D cultures can generally be grouped into (i) suspension cultures on non-adherent and cell-repellent surfaces, (ii) cultures in matrices and substrates, (iii) cultures grown on scaffolds, and (iv) cultures grown with mechanical assistance.

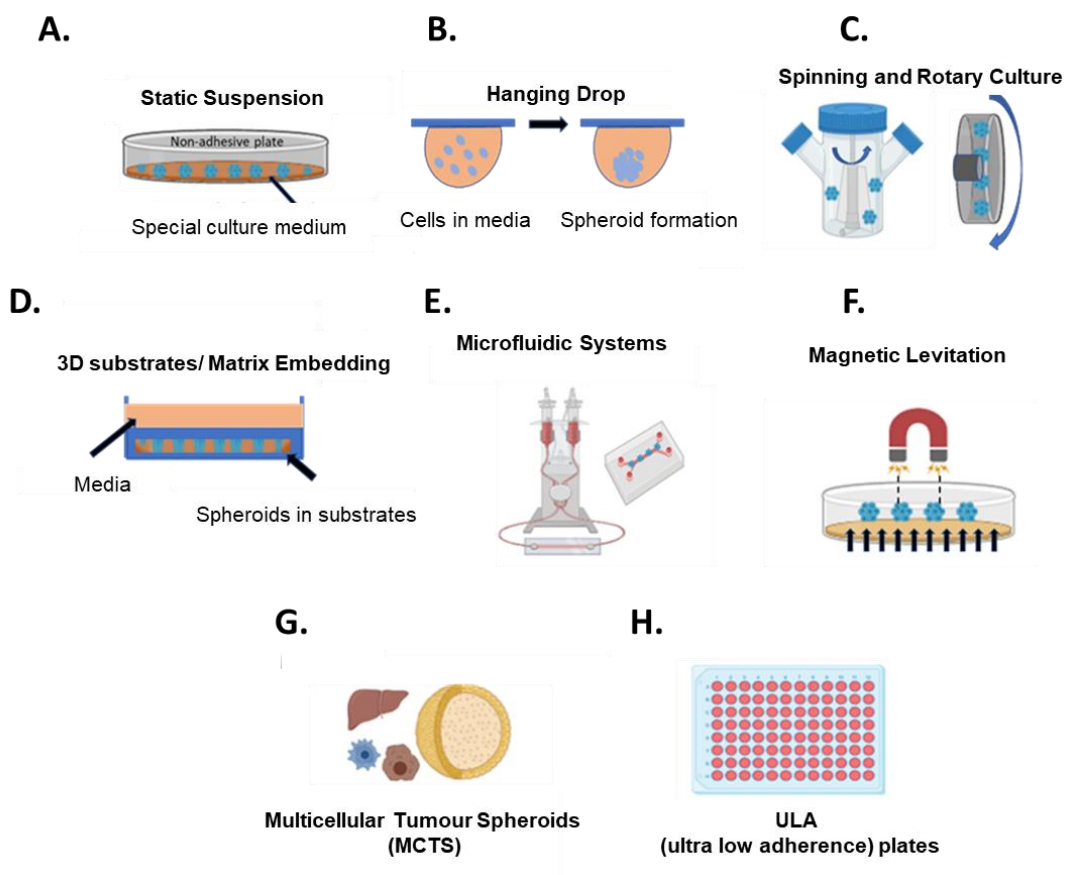


Figure 1.5. Commonly used scaffold and scaffold-free spheroid preparation techniques.

A variety of different techniques for spheroid production exist, each varying in complexity and ease of use. Common techniques include (A) static suspensions, (B) hanging drops, (C) rotary and spinning cultures, (D) substrates and matrices, (E) microfluidic systems, (F) magnet assisted cultures, (G) multi-cellular tumour spheroids and (H) ultra-low attachment surfaces and plates, with each technique causing cells to aggregate and compact into spheroidal microtissues.

Briefly, the general concept surrounding 3D cultures is the formation of 3D structures in which cells form layers, mimicking both the physical and biological structure and function of cells in the *in vivo* environment. In 3D conformation, cells receive stimuli from local environment, in a similar manner to what we observe *in vivo* [194]. In comparison to 2D cultures, which are mainly composed of proliferative cells due to necrotic cells being washed away during culture medium replenishment, 3D spheroids are comprised of cells at varying stages, i.e., proliferating, quiescent, apoptotic, hypoxic and necrotic (Figure 1.6). In a manner that closely mimics *in vivo*, the outer cell layer is strongly exposed to culture medium and essential nutrients, therefore cells remain viable and proliferative. Deeper into the spheroid, cells receive fewer essential nutrients, oxygen and growth factors and therefore tend to be in quiescent or hypoxic states, mimicking *in vivo* tissues (particularly tumours), and deep into the spheroid core, all cells receive little to no nutrients or oxygen, a necrotic core form (Figure 1.6) [196, 197].

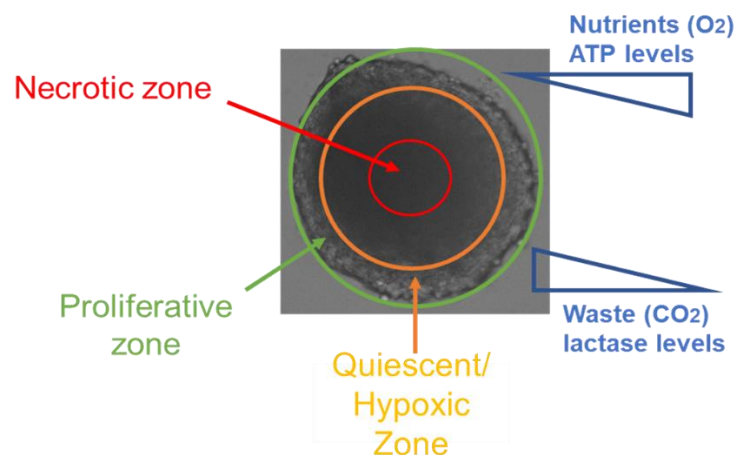


Figure 1.6. Diagram depicting the *in vivo*-like zonation observed in 3D spheroid cultures. In a manner which closely mimics the *in vivo* environment, spheroids show various zones, namely (i) proliferative zone, (ii) quiescent zone, (iii) apoptotic and hypoxic zone, and (iv) necrotic zone.

In 3D environments, *in vivo*-like cell polarity and morphology are also maintained, and can be restored to cells which were earlier cultured in 2D [194, 198]. For example, liver cells show zonation similar to that found *in vivo*, when grown in 3D hepatic cells cluster to form cellular spheroids/microtissues. In recent years, many useful 3D culture systems have been developed and they have quickly become vital experimental tools for many research purposes. Undoubtedly, these advanced systems show great promise in drug and NBM

discovery, by acting as a badly needed bridge between conventional cell culture and *in vivo* animal models. However, much effort and work still needs to be done to assure not only reproducibility, but to also move this technology further, with the establishment of standardized and validated protocols for 3D culture models. More detailed discussion on this topic can be found in the comprehensive 2017 review ‘Modelling Physiological Events in 2D vs. 3D Cell Culture’ [121], and further information about 3D cell cultures, with particular focus on 3D models of the liver is presented below in Section 1.8.

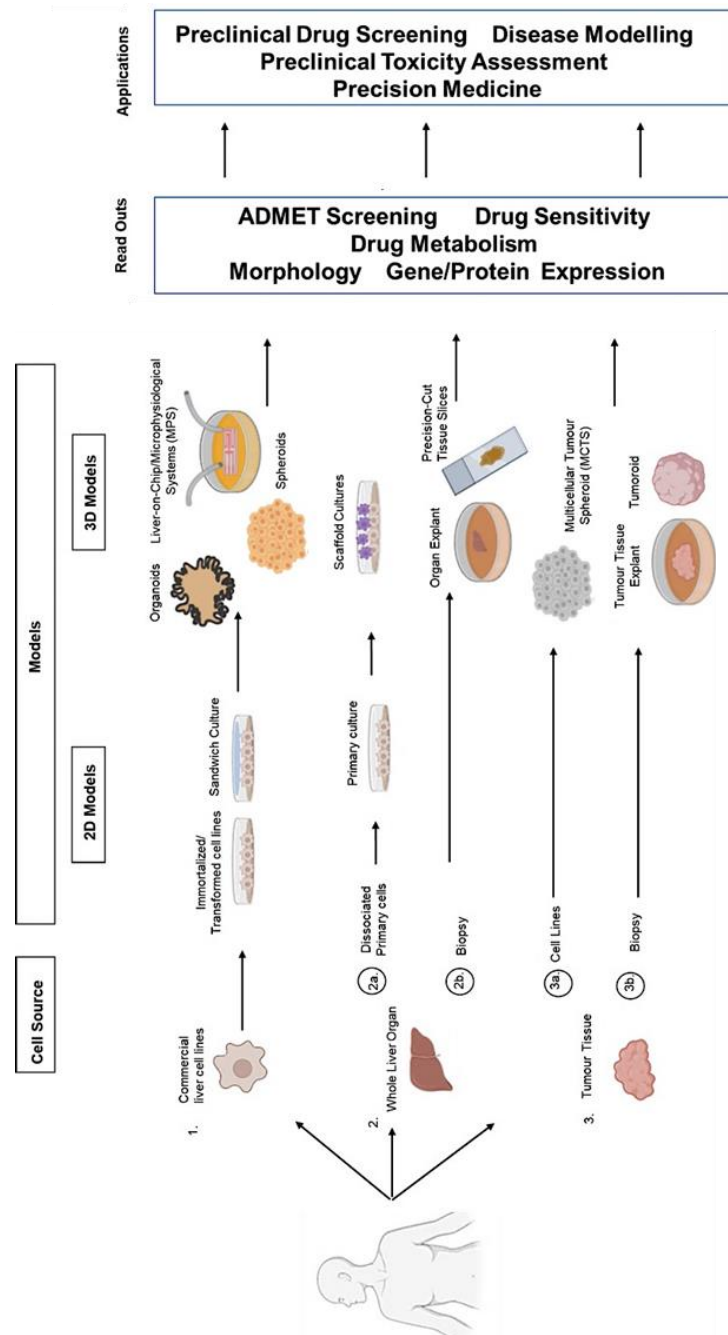


Figure 1.7. Commonly used scaffold and scaffold-free spheroid preparation techniques. Flow diagram illustrates the source of liver cell/tissue, their potential 2D and 3D models formed, their readouts and associated endpoints.

There are a wide variety of methodologies that can be employed for culturing cells in three-dimensions. One of the most frequently used culture techniques is the formation of sandwich cultures, where hepatocytes are cultured between layers of ECM components or culture substrates such as Matrigel or collagen [199, 200]. Whilst not forming complete 3D confirmation with cells (cellular spheroids are not formed), culturing cells in this manner does promote a more *in vivo* like microenvironment. Here, hepatocyte morphology is maintained, with less flattening, increased cell-cell contacts and cellular polarisation [133]. Scaffolds are also widely used for 3D culturing of cells, most of which formed from hydrogels, which have been shown to further improve hepatocyte function in sandwich cultures [201]. Hepatocytes may also be seeded into scaffolds made from either artificial or natural ECM, substrates which allow the cells to migrate towards each other as they would naturally *in vivo* [84]. Culturing hepatocytes as cellular spheroids or multicellular tumour spheroids are also extremely popular methods of enhancing hepatocyte function *in vitro*, and in more recent years a move has been made towards organoid and liver-on-a-chip models. Each of these methodologies will be further discussed below.

1.8 Current *in vitro* 3D models of the liver

Some of the most common 3D *in vitro* models currently available are summarised in Table 1.2.

Table 1.2. Advantages and disadvantages of 3D models of the liver.

	Spheroids/ Multicellular Tumour Spheroids (MCTS)	Scaffolds/ Hydrogels	Organoid/ Tumour	Liver-on- Chip/Microp hysiological Systems (MPS)	Explants/ Tumour Explants	Precision- cut tissue slices (PCTS)
Advantages	• Easy to culture • Mimic <i>in vivo</i> cell-cell environment • Scalable and THP/HTS compliant • Co-cultures possible • Easily extracted for further experimentation	• Hydrogels: <i>in vivo</i>-like 3D interactions • Used to study cell aggressiveness/metastatic potential • Scaffolds: can be combined with functional tests	• <i>In vivo</i>-like complexity • Patient specific • Replicate <i>in vivo</i>-like cell interactions	• <i>In vivo</i>-like architecture • <i>In vivo</i>-like chemical/physical gradients, micro-environment	• <i>In vivo</i>-like architecture • <i>In vivo</i>-like complexity • Useful in disease modelling	• Preservation of 3D architecture • Reflects natural and relevant microenvironment • Hundreds of slices can be obtained from single source • Reproducible • Low cost
Disadvantages	• Size variability • Limited diffusion if large • Necrotic core formation • Agglomeration • Take time to form and exhibit functionality	• Hydrogels: Size/shape variation, hard to reproduce • Difficult cell extraction • Scaffolds: Cells can flatten/adhere to scaffold • Different materials can affect growth	• Complex to culture • Variation • Less amenable to HTP • Needs optimisation/validation • May lack certain cell types/vasculature	• Flow of medium may disrupt cells • Difficult to adapt to HTS • Lack vasculature	• Variation between donors • Difficult to obtain • Complex to culture/expensive to maintain • Lack long-term viability	• Difficult to obtain • Lack long term viability

1.8.1 Ex vivo models of the liver: Precision cut tissue slices, whole organ explants and tumour explants

Ex vivo models include precision cut tissue slices, whole organ explants and tumour tissue explants.

Precision cut tissue slices, or PCTS, can be described as useful and robust *ex vivo* models for toxicity screening, due to their strong *in vivo* relevance and retained liver-specific characteristics, including their conserved multi-cellular architecture. Whilst often generated from rat liver, this methodology can also be employed using human liver samples [202], with human tissues for PCTS normally obtained from either explanted tissue, partial hepatectomies, tissue unsuitable for transplant, or offcuts from surgery (*i.e.*, diseased liver obtained from cirrhotic or fibrotic patients) [203]. By preparing a model in this manner (*i.e.*, by slicing human or animal tissue), ample oxygen and nutrients is supplied to the inner layers of cells. This allows hepatocytes to maintain their structure and morphology, their intracellular polarization for up to five days in culture (15 with optimal conditions). In PCTS, the multi-cellular histoarchitecture of the organ is maintained, including liver-infiltrating immune cells [204]. Several studies have demonstrated that interactions between specific liver cell subtypes are conserved in PCTS, with Olinga *et al.* illustrating the interactions between Kupffer cells and hepatocytes [205]. And moreover, the extracellular matrix (ECM) which is notably absent in hepatocyte 2D cultures, is also conserved, ensuring the regulation of important cellular functions by associated hormones, cytokines and growth factors [206, 207]. PCTS are also useful due to their reproducibility, ease of maintenance, and low cost, making them valuable tools for assessing many elements of various liver diseases. To date, various studies have used PCTS for assessing impact of NBMs. A study from Dragoni *et al.* investigated the uptake and toxicity of AuNPs using rat liver PCTS [206]. Uptake of AuNPs was observed in various liver cells in the slices, including hepatocytes, Kupffer and endothelial cells. A more recent publication from Bartucci *et al.* used murine and, for the first time, human liver slices to investigate *ex vivo* the basic mechanisms of NBM interactions in real time [208]. Real time resolved confocal microscopy was utilised, and flow cytometry undertaken to perform a quantifiable study of nanoparticle distribution and uptake across the variety of different liver cell types recovered from the PCTS. Similar to what is observed *in vivo*, it was found that Kupffer cells accumulated large amounts of NBMs, which interestingly move within the tissue slices to the borders. Despite the advantages and usefulness of PCTS in the pre-screening of NBMs, it is worth noting that this model cannot be used in a high throughput manner, and rapidly lose functionality after

few days in culture, factors which limit their usefulness in NBM pre-clinical assessment [209].

Whole organ explants, which can either be suspended in culture medium or embedded in ECM substrates, also have a variety of uses, mainly in the study of hepatotoxicity and the likelihood of DILI, and offer *in vivo*-like 3D architecture and gene expression which can be maintained up to five days in culture [210]. Whilst conventional studies of the mechanisms and progression of liver disease normally focus on a selection of liver cells and cell culture techniques, whole organ explants offer many advantages over these methods as they not only contain all cell populations in the liver, but also have their 3D architecture maintained. In the past, whole murine liver explants have been used in the study on the effect oxidation has on hepatocarcinoma progression and more recently the viability and functionality of human explants have been studied following exposure to ethanol, as a means of assessing alcohol induced liver injury [211]. Similar to this, tumour liver explants can also be used as 3D *ex vivo* models of human hepatocellular carcinoma (HCC) [212, 213]. These 3D models of hepatocarcinoma, formed from explanted tumorous tissue, which is embedded in collagen or culture medium, are also used as a popular *in vitro* model for drug efficacy screening [214]. A study by Piera *et al.* used liver explants from mice inoculated with a HCC cell line, Hepa1/A1, to study the antineoplastic potential of Citozym, a well-known antioxidant [215]. Despite their many uses and *in vivo* relevance, whole organ and tumour liver explants do have many limitations associated with them. These includes the lack of reproducibility due to inter-donor heterogeneity, lack of viability long term, and issues with availability, rendering them unfeasible models for high throughput (HTP) assays or any chronic toxicity screening [156]. Whilst they are becoming more widely used for modelling disease and for assessing material toxicity, information regards their applicability to NBM pre-clinical assessment is still scarce.

1.8.2 Scaffold methodologies: Culture matrices, substrates and hydrogels

Cell culture matrices, hydrogels and substrates are some of the most commonly used scaffold spheroid formation methods. In recent year, the advancements in material chemistry and material fabrication, and developments in biology have led to the design of these 3D culture materials which represent more accurately the chemistry, geometry and signalling seen in the natural ECM. These materials tend to be porous in nature, and support cell growth, organisation and differentiation both on and within their structure [216]. In 3D matrices, diversity of architecture and material is greater than that of conventional 2D substrates, with

a wide variety of matrices existing, each with different appearance, porosity, surface and mechanical characteristics, and permeability. The applications of 3D culture substrates and matrices depends on the endpoint, with divisions seen between both clinical and *in vitro* 3D modelling approaches. In terms of their clinical applications, they are mainly used in regenerative medicine or tissue engineering, where target materials can be utilised as functional implants, i.e., scaffolds which are designed to be implanted into a patient as a temporary fix to assist with restoration or maintenance of tissue function. Chosen scaffold or matrix must have the appropriate architecture to not only support cell growth, but it must also match the shape of the site it is being implanted into. Scaffolds and matrices used for this purpose must be biodegradable and metabolized by the body without causing any serious systemic or adverse effects, or longer-term issues with the immune system. The second application of culture matrices and substrates, the application most useful here, is their uses in 3D cell culture models. Here, the primary aim is to mimic cell biology at the molecular level, therefore providing us with a significantly greater understanding of actual *in vivo* tissue physiology [217].

A popular method employed for forming hepatic 3D cultures is the use of these matrices and scaffolds for cell growth [84]. One such material, Matrigel™ has proven to be very useful in the culture of hepatocytes for modelling the *in vivo* liver environment, with work undertaken by Molina-Jimenez *et al.* showing that culturing Huh-7 cells in Matrigel™ as 3D models allows prolonged viability (useful for chronic toxicity studies), maintained hepatocyte polarity, and formation of functional transporters [200]. As shown by Bell *et al.* [218], when PHH 3D and 2D cultures were formed from same donors, 3D spheroids grown in Matrigel™ were found to not only be more functionally stable, but also to exhibit greater sensitivity in detecting hepatotoxins. Another natural ECM component, collagen, is also commonly used as scaffold for growing hepatocytes in 3D. When cultured with collagen, many hepatocyte cultures exhibit improved production of urea, albumin synthesis and CYP enzyme activity [219]. In more recent year, the advancements in material chemistry and material fabrication, have led to the design of synthetic 3D culture materials which accurately represent the chemistry, geometry and signalling seen in the liver ECM. These synthetic substrates have been used for studying the liver toxicity of NBMs, with Kotov *et al.* forming highly viable HepG2 spheroids with intact junctions using hydrogel scaffolds. Interactions between hepatocytes and cadmium telluride (CdTe) and gold (AuNPs) nanoparticles, were studied using this model, and it was observed that the toxic effects of both materials were significantly reduced in 3D culture when compared to 2D, with phenotypic changes and the tissue-like morphology identified as the major factors implicated

in these differences. Another scaffold-based culture technique used for growing 3D hepatic cultures and worth mentioning is 3D bioprinting, which uses manufacturing techniques and computer-assisted technology to form 3D structure from cells and biomaterials, with an overall goal of forming functional tissues and organs [220]. The primary advantage of bioprinting is homogenous cell distribution, something which can be difficult when seeding cells onto larger scaffolds. In 2010, the first reported biomimetic liver was formed from HepG2 cells encapsulated in alginate hydrogel and was used for drug metabolism studies [221]. A second notable bio-printed 3D liver model has also been formed which comprised of human-induced pluripotent stem cell (hiPSCs)- derived hepatic progenitor cells, adipose-derived stem cells and HUVECs, with this culture maintained for 20 days, and exhibiting advantages such as enhanced hepatic morphological organization, liver-specific gene expression, increased albumin and urea production, and enhanced cytochrome P450 induction [222, 223]. A more recent publication from Nguyen *et al.* established a novel bio-printed human derived mini-liver formed from HUVECs, hepatic stellate cells (HSCs), and human primary hepatocytes for the *in vitro* assessment clinical drug-induced toxicity [224, 225]. This unique tri-culture 3D model not only allowed for a more relevant assessment of cytotoxicity, but also enabled the measurements of responses specific to each of the cell types.

When using these matrices for 3D cell culture, one of the most important consideration is the endpoint under investigation and other limitations. Matrices and substrates such be chosen carefully, with thought given to the experimental parameters of the study. For example, if the work being undertaken requires a great deal of advanced imaging, i.e. confocal microscopy, matrices such as Matrigel™ may not be optimal due to the difficulty with imaging these 3D cultures and the need for advanced equipment. A further issue, the ability of NBMs to penetrate the dense layers of matrix and scaffold cultures must also be taken into consideration.

1.8.3 Scaffold-free 3D cell spheroids

In recent decades, 3D cell spheroids, also known as microtissues, have gained increased attention in both drug discovery and tissue engineering due to the huge array of advantages they have over existing 2D monolayer cultures. Cell spheroids can be described as small, round clusters of cells which can either self-assemble naturally in non-adherent culture materials [226], with their primary function as an *in vitro* model to provide more physiologically relevant, predictive *in vivo* like data.

A variety of different techniques for spheroid production exist, each varying in complexity and ease of use. Common techniques include ultra-low attachment surfaces and plates, the use of rotary or rocked culture, as well as hanging-drop techniques, with each technique causing cells to aggregate and compact into spheroidal microtissues. Scaffold-free methods are an advantageous methodology to form spheroids, as they allow cells to self-aggregate to form microtissues as cells would do in an *in vivo* situation, they avoid the formation of phenotypes triggered by complex interactions between cells and culture substrates/matrices, they allow formation of direct cell-cell contacts, production of tissue-specific ECM, and maintenance of viability for long periods of time, overcoming some the most common limitations of conventional 2D and scaffold-based 3D models.

Ultra-low attachment 96-well, or ULA plates (and ULA surfaces in general) are popular scaffold-free methodologies. Cost effective and easy to use, spheroids can be produced using ULA plates by a ‘forced floating’ method. Here, round-bottomed cell culture plates with low-adhesion properties or hydrophilic polymer coatings are used for quick and easy spheroid production. ULA plates form one single, uniform spheroid per well, with a major advantage of this method being the possibility of automation and its suitability for high-throughput screening [227]. Another widely used method of spheroid formation is the hanging drop system, undertaken using either a hanging drop plate setup (e.g., InSphero™ system), or by using inverted petri dishes. For the InSphero™ set up, a system recommended for use with primary cells or co-cultures, cells are plated into a GravityPLUS™ hanging drop plate and left for 4 days to aggregate, before being dropped into the companion GravityTRAP™ ULA plate for long term cultivation. Whilst this method forms spheroids with great consistency and accuracy, the companion ULA plate is less ideal for microscopy and imaging, with spheroids needing to be transferred to microscopy slides for further analysis.

Scaffold-free 3D spheroids provide vastly more representative models when compared to their 2D counterparts, as they can closely mimic the microenvironments, cell/cell and cell/matrix interactions, and biological process which occur in an *in vivo* scenario. In previous studies, it has been found that when spheroids which are formed from primary hepatocytes or immortalized hepatic cell-lines, enhanced *in vivo* liver like structure and functionality are observed [228]. They show a high degree of both morphological and functional differentiation, traits which are characteristic to the *in vivo* cell environment. The levels of both protein and genes expressed in spheroids closely reflects the levels found in *in vivo*. By using scaffold-free 3D spheroids, we also have a better understanding of the toxicity of compounds/materials, as these models allow reproducing drug penetration and

proliferation gradients, which cannot be mimicked in 2D cultures and scaffold-based 3D models. Thus, scaffold-free spheroids can act as better predictors of both drug and NBM effects in humans. Also, thanks to their long lifespan, 3D spheroids can be useful for repeated dose or chronic toxicity testing, a major advantage over conventional methods [227].

In the area of scaffold-free hepatic cell spheroids, most common models are grown from a combination of immortalized hepatic cell lines (i.e., HepG2, Huh7, and C3A), primary human hepatocytes (PHH) and non-parenchymal cells including stellate cells (HSC), liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KC). Scaffold-free hepatic spheroids have been used to model many disease states, ranging from steatosis, useful for identifying novel drugs for treating NAFLD [229], to fibrosis [230]. Scaffold-free hepatic spheroids have also proven useful tools in the study of drug-induced liver injury (DILI). As DILI cannot be accurately predicted using animal models, *in vivo* animal safety tests are normally combined with assessments in human *in vitro* systems. Using spheroidal cultures of PHH which can be maintained in culture for long term (>5 weeks) have been extremely useful in this area, with Vorrink *et al.* evaluating the hepatotoxicity of 123 drugs using this model [231]. Overall, it has been shown that spheroids formed from varying hepatocyte cell lines have enhanced liver-specific function when cultured in 3D, overcoming many caveats and inadequacies seen with conventional immortalised hepatocyte 2D cultures, with sustained Phase I and II enzyme expression, increase albumin and urea secretion as well as expression of various liver specific materials. Canalicular like networks are also observed, illustrating hepatocyte polarisation in culture [232, 233]. It has been shown than when HepG2 cells are cultured as scaffold-free 3D spheroids, they secrete more albumin and urea, and over-express many genes which play roles in xenobiotic and lipid metabolism, and exhibit more *in vivo*-like liver structure [234]. Various studies have used co-cultures of hepatocytes with Kupffer cells to form scaffold-free 3D spheroids. These studies have shown that the *in vitro* models formed have enhanced liver-specific structure and function. Endothelial cells have also been integrated within hepatocyte/Kupffer spheroid co-cultures, demonstrating viability up to 35 days, with enhanced expression of transporters and hepatotoxin sensitivity also observed [232]. Scaffold-free spheroids formed from hepatic stellate cells and hepatocytes have also been developed, albeit these models have been investigated in less detail, triggering some of the work carried out within my Ph.D. project. In the scientific literature, hepatic stellate cell/hepatocyte spheroids, have been reported to have enhanced CYP activity and expression, with a sustained increase in albumin production up to 30% for up to 2 months after seeding [226, 235]. Co-culturing hepatocyte cell lines in 3D is also useful for providing

insight into the organisation of various cells of the liver, and their reorganisation over time [84].

1.8.4 Multicellular tumour spheroids (MCTS)

A specific type of scaffold-free 3D spheroids are MCTS. MCTS are one of the best characterised and more widely employed 3D organotypic models of cancer that exist today. They are formed from homogenous tumour cells or co-cultures on non-adherent surfaces, where cells aggregate and compact naturally [156, 236]. These models can mimic the morphological, functional and physiological cellular properties of tissue and bear greater resemblance to both avascular micro metastases or intravascular regions of solid, large tumours, displaying intermediate complexity between conventional 2D models and *in vivo* solid tumours [137, 227]. Since their introduction in the early 1970s by Sutherland and co. [237, 238] they have been used as a means of gaining greater insight into the therapeutic challenges faced in the clinic with regard drug resistance, cell/cell and cell/matrix interactions and metabolic and proliferative gradients [227]. Widely used in the study of human hepatocellular carcinoma (HCC), hepatic MCTS can be used to screen chemotherapeutics which can help provide more informative data for clinical drug applications [239]. MCTS have been shown to tolerate greater doses of chemotherapeutics than their 2D monolayer controls, possibly due to the hypoxic environment presented within the spheroid, a condition which activates the extracellular signal-regulated kinases (ERK) pathway, an essential pathway for tumour cell proliferation in patients [240]. In recent years, the application of MCTS in the drug discovery field has grown exponentially, allowing the screening of a wide array of varying chemical compounds [241-243]. Despite the labour-intensive handling, relatively large costs associated with their formation and culture and the instrumental limitations associated with MCTS, the benefits greatly outweigh the caveats, and they are currently considered an appropriate and useful 3D model for toxicity screening for oncological drugs [241, 244]. A detailed overview of how MCTS have been used for the assessment of nanoparticles can be found in a review from Lu and Stenzel entitled 'Multicellular Tumour Spheroids (MCTS) as a 3D *in vitro* evaluation tool of Nanoparticles' (2018) [245].

1.8.5 Organoids

In recent years liver organoids have emerged as a highly relevant and useful alternative *in vitro* model with excellent potential for disease modelling and drug/NBM screening. This model has been found to be advantageous over conventional *in vitro* models due to their long-term genetic stability, *in vivo* like organization and their ability to maintain the cellular crosstalk and behaviour of their primary cell counterparts [246, 247]. Organoid cell culture can be described a collection of several cell types that develop from induced pluripotent stem cells (iPSCs), pluripotent embryonic stem (ES) cells, or organ-specific adult stem cells (aSC), both healthy and diseased [137]. These models not only display many organ-specific functions, but also mimic structural organization, and self-organize through cell sorting, in a similar manner to organogenesis in an *in vivo* environment [248, 249].

3D hepatic organoids are advantageous over conventional *in vitro* models as they have long-term genetic stability, an *in vivo* like organization and the ability to maintain the appropriate cellular crosstalk and behaviour of their primary cell counterparts [250]. It has been proposed that hepatic organoids formed from a co-culture of Kupffer cells, hepatic stellate cells and various inflammatory cells may be useful for studying how non-alcoholic fatty liver disease (NAFLD) transitions to non-alcoholic steatohepatitis [251]. These functional models also serve as useful platforms to address a wide variety of research questions, ranging from hepatic development and regeneration, to metabolism and detoxification, and are also extremely representative model diseases of the liver including non-alcoholic steatohepatitis (NASH) and NAFLD [247], acting as an excellent resource for studying the human liver in ways that were previously very difficult.

As detailed by Mun *et al.*, hepatic organoids help overcome the limitations of conventional 2D models as they have been shown to express Phase I drug metabolism and Phase II detoxification enzymes. In this study, following treatment with omeprazole, CYP1A2 and 3A4 induction was observed in hepatic organoids. Additionally, when treated with hepatotoxic drugs like troglitazone and APAP in conjunction with 2D hepatocyte cultures, hepatic organoids exhibited a markedly higher sensitivity than the 2D cultures. These organoids have been extensively investigated and applied to liver organoids as most recently reported in the comprehensive review works of Prior and co-workers entitled ‘Liver organoids: from basic research to therapeutic applications’ [247] and ‘Next Generation Liver Medicine using Organoids Models’, also published in 2019 [252].

Organoid models have been applied to the pre-clinical assessment of the biocompatibility and toxicity of NBMs, with complex organoid systems being developed to help gain a greater understanding into the multifaceted interactions that occur between NBMs and varying cell types and tissues [253-255]. One study undertaken by Kermanizadeh *et al.* assessed the hepatotoxicity of silver nanoparticles (AgNPs), among others, using hepatic organoids formed from human primary hepatocytes and NPCs. Following organoid characterisation, various parameters including cytotoxicity and genotoxicity, were assessed following NBM exposures [256]. Hepatic organoids have also been applied to the assessment of complex NBM systems, such as the liposomal DNA origami nanosystem (LSTDO) designed by Palazzolo *et al.*. Doxorubicin was loaded inside the LSTDO, and the system tested in murine hepatic organoids, to determine if such a system would be clinically relevant and aids to improvements on the current cancer treatment. Biocompatibility was ensured by undertaking toxicity screening using the mouse-derived hepatic organoid model, and it was found that the DNA origami internalization into liposomes negated many of the toxic effects of free DNA origami [257].

Despite the major advancements in the field of hepatic organoids in recent years, challenges are still present in this area, specifically with the accurate recapitulation of the *in vivo* ECM. Limitations also occur due to the lack of native microenvironment, an issue which inhibits the study of interactions between stem cells and their niches, a distinct lack of appropriate growth factors and an incapability to appropriately model immune response. Possible means of overcoming these issues may be organotypic co-cultures, or the application of microfluidics to these models.

1.8.6 Liver-on-a-chip/Microfluidics

A primary disadvantage of conventional *in vitro* models is the issue of providing a constant flow of culture medium/nutrients to the cells, and the artificial way medium is renewed sequentially, to either keep cells alive and healthy, or to introduce drugs or materials for toxicity screening [258]. Some of the most recent 3D culture advances have been achieved in an attempt to overcome these limitations, with organ-on-a-chip microsystems developed and capable to reproduce many key structural, functional, biochemical and mechanical features of living organs, in a single, small device [259]. Microfluidics has also been utilised in liver biology field. Here, microfabricated structures have been formed within microfluidic channels that closely resemble the endothelium which separates hepatocytes from the liver sinusoid in the human liver, and with a constant flow of nutrients, metabolites and oxygen

maintained through the microfluidic channel, something which cannot be replicated in 2D models, and something which generally improves cell viability, culture lifespan, and metabolic performance [260, 261]. Liver-on-a-chip models have been shown to maintain their metabolic competencies for over one week, with key hepatic characteristics visible, including formation of bile canaliculi [262, 263]. Due to the constant flow of low concentrations of nutrients, metabolites and oxygen, in microfluidic systems a more *in vivo*-like system can be maintained, improving liver specific functions in hepatocytes [233].

One example of liver-on-chip for *in vitro* screening of hepatotoxins is the 3D HepaTox Chip. Designed by Toh *et al.* [264], this is one of the first demonstrations of microfluidic system that could accurately predict hepatotoxicity, where data obtained using this model strongly correlates to *in vivo* data [264]. In this model, hepatocytes maintain a comparable, if not higher, concentration of phase I and II metabolic functions than 2D cultures. After 24 hours in culture, basal CYP 1A1 and CYP1A2 levels are approx. 3-fold higher in the HepaTox Chip [264]. Another study undertaken using C3A cells and microfluidic devices demonstrated that multiple liver specific functions including synthesis of albumin, gluconeogenesis and ureagenesis were at almost comparable levels to those found in primary hepatocytes [265]. Other than the HepaTox Chip and simpler microfluidic systems, various more sophisticated systems exist, including hollow-fibre bioreactors. Here, cells are seeded on complex scaffold with a constant flow of culture medium present. One such study from Gerlach *et al.* successfully cultured human primary hepatocytes and NPCs using this method, with viability maintained for at least 3 weeks and vascular cavities and bile-duct like channels were also visible [266]. In more recent years, more advanced human liver-on-chip model consisting of multiple hepatic cells such as hepatocytes, LSECs, KCs and HSCs was developed by Jang *et al.* [267]. Over the years, many microfluidic systems have been developed commercially, with many of them available through companies such as Mimetas, TissUse and Emulate, among others, therefore facilitating their use as emerging hepatic technologies. These novel microfluidic hepatic models have also acted as a new platform for testing NBMs.

Due to the fact NBMs exhibit different behaviour depending on whether they are under static or flow conditions, the development of dynamic flow systems are extremely important for accurately determining NBM-induced toxicity [258, 268]. Notable examples of this include a study from Li *et al.* who developed a 3D microfluidic hepatocyte chip for assessing the hepatotoxicity of superparamagnetic iron oxide nanoparticles (SPIONs). For this model, primary rat hepatocytes were used to fabricate a three-layer chip which tested SPIONs in both short- and long-term culture [269]. A second study from Liu *et al.* incorporated

electrospun fibres in a PDMS microfluidic chip and used this device to culture primary hepatocytes for assessing AgNP hepatotoxicity. Here, hepatocyte behaviour was studied, and it was determined that by using this platform under optimised flow conditions, specific hepatocyte functions including polarity and biliary excretion were restored and maintained for up to 15 days. Hepatocytes under a flow rate of 10 $\mu\text{L}/\text{min}$ also produced sensitive and consistent toxicity responses to AgNP, demonstrating the applicability of this model for the *in vitro* toxicity screening of NBMs [270].

Liver-on-a-chip models can also be incorporated into more complex microfluidic systems called micro physiological system (MPS), systems where several organs-on-chips are connected [271]. The MPS concept was born from the inability of 2D monocultures of immortalized cell lines or *in vivo* animal studies to sufficiently recapitulate the dynamic of drug-drug, drug-organ and drug-organ-organ interaction in humans. Initially, MPS were developed as a mean for increasing efficiency, speed and safety in the pre-clinical development and assessment of pharmaceuticals. More recently, MPS have been utilized to assess efficacy and safety of NBMs in one single, compact *in vitro* platform, ultimately advancing the translation of these materials [272]. A recent example this can be seen with a GI/liver MPS incorporating co-cultures of Caco-2/HT29-MTX and HepG2/C3A liver cells which has been successfully applied for investigating first pass metabolism of high doses of polystyrene nanoparticles intended for daily human consumption [273]. This study determined that despite low nanoparticle permeability being observed across the GI barrier, the single nanoparticles and small clusters which did in fact pass through induced aspartate aminotransferase (AST) release, indicating liver injury. Overall, sophisticated MPS are a unique opportunity, offering fundamental understanding of NBM effects in the human body [274].

However, despite the many benefits associated with microfluidic models, they are not without fault due to their complex nature. Issues like disaggregation and loss of cells can occur to the constant perfusion of cell culture medium, particularly as cells proliferate and reach confluence. Furthermore, the effect of sheer stress on cultured cells has yet to be fully elucidated in some cases [275]. Finally, the large set up and maintenance costs associated with these models and systems, as well as the large numbers of cells needed to build them and the associated issues with their handling and data analysis are also limiting factors for the uptake of these complex platform technologies for the high-throughput screening of NBMs.

1.9 3D models in the pre-clinical screening of NBM hepatotoxicity

As detailed previously, emphasis is currently being placed on more advanced *in vitro* methodologies for assessing interactions and possible effects of NBMs [276], 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 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 [277]. 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 [276]. 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 [278]. In general, liver spheroid cultures tend to be more resistant to the impact of NBMs when compared to 2D cultures. A variety of things contribute to the decreased toxicity of NBMs in 3D models. Firstly, as compact spheroids form tight and gap junctions, only hepatocytes which are on the spheroid periphery have access to the NBMs, whereas the cells deep within spheroid core often cannot be reached by NBMs. Spheroids lack in fact capillary veins, which facilitate easy transport of NBMs to spheroid core. In contrast, 2D hepatic monolayers easily absorb NBMs due to all cells being in contact with treatment. Secondly, highly differentiated hepatocytes have a relatively low endocytic ability as compared to hepatocytes cultured in 2D [279].

3D models can be useful in the prediction of NBM tumoral penetration capacity, therefore assisting in the optimisation of their physicochemical characteristics [105, 278, 280]. Liver (HepG2) spheroids have been used for example by Jiang *et al.* to underpin how a NBMs charge effects its uptake. Here, negatively charged polystyrene NPs (ζ potential of -34.9 mV) were better able to penetrate spheroids than positively charged one (ζ potential of 9.4 mV) [281]. Spheroids have also been used to fine tune the shape of NBMs, with Zhao *et al.* demonstrating that spherical NBMs exhibit greater uptake than their rod-shaped counterparts [282]. Fledderman *et al.* have also used HepG2 spheroids cultured as 3D spheroids to assess penetration and uptake of SiO₂ nanoparticles in order to obtain a greater understanding of nanoparticle uptake in solid tissues [283].

3D spheroids have been used in the screening of the potential of NBM therapeutic efficacy [278, 284, 285]. Due to the structural similarities between spheroids and *in vivo* solid tumours and tissues, the efficacy observed in 3D models are normally reduced when compared to 2D cultures. Other studies have used hepatic spheroids in the pre-clinical assessment of NBM cytotoxicity. Elje *et al.* have used HepG2 spheroids to assess the cytotoxicity of titanium dioxide (TiO₂), silver (Ag) and zinc oxide (ZnO) nanoparticles [284]. Due to the structural similarities between spheroids and *in vivo* solid tumours and tissues, the efficacy observed in 3D models are normally reduced when compared to 2D cultures [278]. In a study by Dubiak-Szepietowska *et al.*, complex multicellular liver spheroids were developed, using hydrogels including transglutaminase-cross-linked gelatin, collagen type 1 and growth factor depleted Matrigel™, as human-relevant models for nanoparticle toxicity assessment *in vitro*. AgNPs, TiO₂s and SiO₂s were assessed using 2D HepG2 monolayer cultures and 3D HepG2 spheroids, with toxicity of each nanoparticle differing significantly between culture types, suggesting that cellular environment significantly influences cell sensitivity to nanoparticles. These results suggest that 3D cell culture models can improve on the predictive outcome for nanoparticle pre-clinical assessment and toxicity screening [286]. Carbon based NPs such as SWNTs have also been studied in 3D cultures, with these particles cytotoxic in both 2D and 3D cultures, however to a very different extent in the 3D tissue mimetic model used [287].

In the last year, a small number of studies have also used hepatic 3D spheroids in the assessment of a largely overlooked area of NBM pre-clinical assessment - genotoxicity. HepG2 spheroids were used to assess DNA damage induced by titanium dioxide (TiO₂), silver (Ag) and zinc oxide (ZnO) nanoparticles using the alkaline comet assay. In this study it was found that differences in toxic responses between 2D and 3D culture can be different, with 3D cultures appearing more resistant when exposed to Ag-NPs. TiO₂-NPs however induced no effects, with ZnO-NPs induced a strong cytotoxic effect in both 2D and 3D models. Higher variability was also observed in 3D spheroids, emphasising the need to optimisation. Llewellyn *et al.* have adapted the micronucleus assay for use with HepG2 spheroids. Here, titanium dioxide (TiO₂) and silver (Ag) were evaluated following both acute and long-term exposures in the HepG2 spheroids, and subsequent genotoxic potential was analysed using the micronucleus assay. It was found that the 3D hepatic model could be suitably applied to the micronucleus assay, where a variety of realistic exposures scenarios are evaluated

A further study from Conway *et al.* have also adapted the micronucleus (MN) assay for use with hepatic spheroids, using HepaRG and HepG2 spheroids to assess DNA damage induced

by ZnO nanoparticles [288]. It is worth noting that each of these studies however only assesses engineered nanoparticles (with the exception of AgNPs) and to date, to the best of my knowledge, no study has adapted hepatic spheroids to the testing of liposomes, AuNPs or PACA NBMs (for more information on this, please refer to Chapter 4 - Discussion).

A small number of studies to date have used hepatic spheroids to assess NBM uptake/penetration. Chelobanov *et al.* have used HepG2 spheroids to assess the uptake and interactions of 12nm AuNPs coated in both bovine serum albumin (BSA) and polyethyleneimine (PEI). Here it was found that HepG2 spheroids did not take up bare AuNPs but were readily penetrated by AuBSA-NBMs and AuPEI-NBMs, emphasising the importance surface characteristics have on NBM uptake and biodistribution. England *et al.* have assessed various sized AuNPs (50 nm – 100 nm) in HepG2 spheroids, 2D HepG2 monolayers and *in vivo* animal studies [289]. Here it was found that NBM penetration increased as incubation time was extended, with the smaller AuNPs (50 nm) penetrating spheroids up to 4 times more than their 100 nm counterparts. These results were in agreement with other studies that suggest penetration of NBMs in spheroids is limited to those less than 100 nm in size [290]. Using HepaRG spheroids to stimulate liver tissue, Senyavina *et al.* studied the effect of 10nm AgNPs, finding that the 3D model was less sensitive when compared to 2D monolayer culture treatments, with viability being significantly higher than observed in 2D [285]. One of the main hurdles for the success of tumour-specific liposomes is inability to sufficiently penetrate tissues. Zhang *et al.* have used HepG2 spheroids to assess the penetration of liposomes equipped with a cell penetrating peptide, BR2, conjugated to their surface, and encapsulation the anticancer drug cantharidin (CTD), in an attempt to create a system which may target HCC cell more effectively and efficiently [291]. A handful of studies have also used hepatic spheroids in the pre-clinical assessment of metallic NBMs, with Miyamoto *et al.* utilising HepG2 spheroids to assess uptake and localisation of various magnetic IONPs. Here, it was confirmed that IONPs were incorporated deep into spheroids, with NBMs observed in both planar and vertical directions. IONPs were localised to the lysosomes of spheroids, in a similar manner to what is observed *in vivo* [292].

A very recent development with regard hepatic spheroids is their application to magnetic hyperthermia, and to date a very small number of studies has probed their potential in this area, including Peng *et al.* who have adapted HepG2 spheroids to the assessment of triphenylphosphonium cation-superparamagnetic iron oxide nanoparticles (TPP-SPIONS). This study revealed that spheroids, in comparison to cell monolayers, better reflected the *in vivo* tumour environment, with spheroids less responsive to TPP-SPIONS during magnetic hyperthermia treatment [293].

Despite promising results in these studies, use of hepatic spheroid models in the testing of NBMs is relatively uncommon, and the application of hepatic spheroid models into the pre-clinical assessment cascade for NBMs is proceeding very slowly. Whilst alternative methods for toxicity screening using reconstructed skin and cornea are validated towards whole organ *in vivo* responses, [294], including a skin co-culture developed in the 90s, comprising of human dermal fibroblasts and epidermal keratinocytes [295], and a 3D human epidermis model (EpiDerm™) for identifying the hazardous potential and genetic toxicity of various irritant substances, including many NBMs used in sun creams (OECD TG 439) [296-298], including a skin co-culture developed in the 90s, comprising of human dermal fibroblasts and epidermal keratinocytes [295], and a 3D human epidermis model (EpiDerm™) for identifying the hazardous potential and genetic toxicity of various irritant substances, including many NBMs used in sun creams (OECD TG 439) [296-298], and are written into guidelines and adopted by various regulatory agencies, to date, no validated 3D model of the liver exists for drug or NBM screening alike. Although 3D hepatic models have been developed and are widely investigated for drug screening by many research groups, pharmaceutical companies and contract research facilities (CROs), there are many barriers for their commercialisation and market development. These issues include quality and availability of source material, government regulations on drug safety, public opinion on sample donation, as well as issues with import and control of cell products, something which varies greatly between countries [279]. As discussed previously, freshly isolated PHH are currently the gold-standard cell type for drug metabolism studies and hepatotoxicity assessment *in vitro*. Despite their many advantages however, i.e. abundance when extracted from liver samples, high quality and functionality, their uses in 3D hepatic models are limited due to the high costs they incur and disposability (PHH are one-time use only). These issues prevent the development of 3D human PHH liver models by smaller research institutes and universities, with development and validation of these models having to be undertaken by larger pharmaceutical companies, i.e. Genentech, Astra Zeneca etc., or CROs whose speciality is hepatotoxicity assessment using 3D model, e.g. InSphero™, Emulate, Inc., BioPredic. Whilst the HepaRG cell line may act as a surrogate for PHH in hepatotoxicity and metabolism studies in 3D, the additional requirement of the extended culture time for this cell line (two to three weeks for differentiation) introduces more variability between lots and increases the risk of contamination, greatly impeding their extensive use in scientific research. A recent breakthrough in the area of hepatotoxicity screening has been with the company INDIGO Biosciences, who have developed a propriety Upcyte®, a cryopreserved luminescent reporter hepatocyte that expresses luciferase (proportional to cell viability).

However due to issues with intellectual properties (IP), this modified cell line cannot be used during drug development. Some companies also prefer to use in-house methods instead of 3rd party tools in hepatotoxicity screening, so that if a drug or material reaches commercialization the developers can't claim part of profit. Therefore, the limitation of PHH and their irreplaceability allow a very small number of research institutes to use 3D hepatic models for assessing toxicity of drugs. This in turn has a knock-on effect for their uses in the pre-clinical screening of NBMs [279]. However since regulatory guidance's on using cornea-like epithelium and reconstructed human epidermis have been announced, it is only a matter of time before the application of 3D hepatic liver models in drug and NBM screening alike is proposed and pushed forward and become an internationally agreed screening method. This will be assisted by the numerous European projects currently underway whose goal it is to develop, standardise and validate complex 3D liver models for NBM screening, including the Horizon 2020 projects PATROLS (Physiologically Anchored Tools for Realistic nanomaterial hazard aSessment, Grant agreement ID: 760813), and REFINE, a regulatory science framework for nano(bio)material-based medical products and devices (Grant agreement ID: 761104). As mentioned previously, REFINE are also the funders of this project.

To summarise, the liver is the largest solid organ in the body, which carries out a wide variety of complex and important functions, ranging from homeostasis of nutrients to clearance of xenobiotics. As induction of toxicity is one of the main reasons for which NBMs are either rejected or removed from the market, sensitive hepatotoxicity screening methods are vitally important in drug development for hazard assessment of NBMs. Due to the reductionist environment presented in 2D liver cultures, and the overall metabolic differences overserved between animals and humans, it is suggested that alternative methods based on 3D cultures may be a vitally important *in vitro* tools for bridging the gap between traditional 2D screening and animal models. As a result of this, in recent years hepatotoxicity screening methods in 3D models are being developed and commercialized for drug screening. In spite of the previously mentioned obstacles to model validation and widespread acceptance that have hindered the rapid development of these models, the standardisation, validation and acceptance of reconstructed skin and cornea models, which are now enacted in OECD guidelines and used worldwide, is a positive outcome and good news for the area of 3D models. With greater support from governments, funding agencies and non-profit organizations (such as IQ MPS, a science-focused collaboration between various pharma and biotech companies whose goal it is to challenge the gaps between current *in vitro* and *in vivo* models and design complex models beyond 2D), there is no doubt that in the coming

years 3D liver models will eventually be adopted by global regulatory agencies, ultimately providing crucial information on the safety of drug compounds and NBMs. It is likely that the newest generation of hepatic 3D models will not only display liver-specific architecture, viability, functionality and physiological responses similar to that which is observed in the native liver, but also contain various biocompatible scaffolds which can increase efficiency of model formation and reduce batch-to-batch variation. To satisfy the strong need for more biologically relevant tissue-mimetic 3D models for drug and NBM screening, it is critical for material scientists, toxicologists, biologists, engineers and regulators to work together to develop these 3D models and to overcome the multiple challenges face with them. Whilst 3D spheroids are much more realistic models of the human liver when compared to 2D monocultures, work still needs to be done to fully optimise these models as pre-clinical assessment tools. Despite the usefulness of 3D models in the preclinical assessment of NBMs, no standardised or validated 3D cell culture model, neither healthy nor in diseased state, has been approved for use in their pre-clinical assessment, and the need for more advanced models is still unmet. With all of the above being said and with this unmet need in mind, the overall aim of this project is to develop a hepatic spheroid model, with liver-specific structure and function, which may be incorporated into the pre-clinical assessment cascade for NBMs, bridging the gap between reductionist 2D *in vitro* models and *in vivo* animal models.

1.10 Project Goal, research questions and project specific aims

1.10.1 Project hypothesis and overall goal

Based on the scientific evidence presented in the previous sections, the working hypothesis here is that 3D hepatic spheroid models are a better predictor of NBM safety than a traditional 2D monoculture model. Thus, the overall goal of this research is to assess whether hepatic spheroids could be applied to the assessment of NBM hepatotoxicity to increase the predictive value of the preclinical assessment cascade currently in use. This is further described in the research question, which is addressed by tackling three separate aims, which are detailed below.

1.10.2 Research question

- Can 3D hepatic models successfully fill the knowledge gap between existing *in vitro* screening methodologies and *in vivo* animal models for predicting human-specific hepatotoxicity in response to NBM exposure?

1.10.3 Project specific aims

1. To establish an effective methodology for growing viable and tissue-mimetic 3D mono- and multi-cellular hepatic spheroids (Chapter 2)
2. To determine the biological relevance of the 3D hepatic spheroids developed (Chapter 3). Here, the term “biological relevance” indicates the ability of 3D hepatic cultures to provide qualitative information on NBM accumulation in the human liver that is comparable to that predicted by *in vivo* animal studies.
3. To determine the functional relevance of 3D hepatic spheroid models developed, when assessing NBMs’ liver toxicity (Chapter 4). Here, the term ‘functional relevance’ refers to the ability of 3D cultures to exhibit cytotoxicity and genotoxicity profiles that are comparable to those detected in *in vivo* animal studies.

Chapter 2

Development of tissue-mimetic 3D hepatic
multicellular spheroid models

Chapter 2 - Development of tissue-mimetic 3D hepatic multicellular spheroid models

Aim

To establish an effective methodology for growing viable and tissue-mimetic 3D multicellular hepatic spheroids

2.1 Chapter summary

As discussed in Chapter 1, predictive *in vitro* liver models are of vital importance for the safety screening of NBMs. Therefore, during my PhD project I have optimised the protocols for forming 3D multicellular hepatic spheroids that are described within this chapter, with the aim of growing viable and tissue-mimetic hepatic models for the predictive *in vitro* testing of NBM hepatotoxicity. HepG2 cells, an epithelial-like, non-tumorigenic cell line derived from human hepatocarcinoma, were used as they are the most widely studied and well characterised human hepatic cell line. Various studies have demonstrated that HepG2 cells exhibit a CYP1A2 and CYP3A4 expression similar to that found in primary human hepatocytes. For this reason, HepG2 cells are widely used to study toxicological responses. Both mono- and multi-cellular spheroids were formed. The latter was formed by HepG2 cells co-cultured with the human hepatic stellate cell line, LX-2. Since scaffolds have been shown to cause a marked influence on the behaviour of various cell types, non-adherent, scaffold-free culturing conditions were preferred for achieving the project aim. In detail, two commercial culturing plates were tested for their suitability to form hepatic spheroids: (i) CellStar[®] cell repellent plates, and (ii) the InSphero[™] hanging drop system. These plates were chosen as they are commercially available and easily sourced, standardised and high throughput in nature, whilst also known for producing reproducible spheroids.

Characterization of the size, morphology, growth of the spheroids formed, as well as of their structural and functional properties, was carried out. Structural parameters included internal spheroid structure as judged by H&E, spheroid viability over time, determined by ATP quantification, and formation of secondary structures, i.e. bile canaliculi. Functional parameters included production of human serum albumin, secondary structure functionality (e.g. functional bile canaliculi, determined by assessing movement of the membrane permeable substrate CMFDA out of cells) and sensitivity of HepG2 spheroids to a panel of human hepatotoxins. A variety of human hepatotoxins were chosen, namely fialuridine, diclofenac, trovafloxacin, and acetaminophen. These materials were chosen due to their

varying mechanisms of toxicity, with it being suggested that fialuridine and diclofenac exhibit toxicity by damaging mitochondria, and that some hepatotoxins such as diclofenac cause transporter dysfunction or loss of hepatocyte structural integrity, something which ultimately causes toxic substances to build in hepatocytes due to issues with bile secretion. Because it is often metabolites of certain compounds which can cause damage to the liver, either by being directly hepatotoxic or from the formation of adducts with proteins in the liver, acetaminophen was also assessed. Trovafloxacin was chosen as other studies have suggested that immune response may also play a role in hepatotoxicity, by heightening the response to hepatotoxins.

2.2 Materials and Methods

Consumables were purchased from Sigma Aldrich, Ireland, unless otherwise stated.

2.2.1 Cell Culture

Human liver hepatocellular carcinoma (HepG2) cells and human immortalized hepatic stellate cell (HSC) line LX-2 were used in this study. HepG2 cells were kindly supplied by SINTEF (Norway). LX-2 cells were kindly supplied by Professor Paul Spiers, Pharmacology and Therapeutics, School of Medicine, TCD. LX-2 cells were cultured in RPMI medium, supplemented with 10% FBS and 1% Penicillin-Streptomycin, at 37 °C and 5% CO₂; whereas, 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; LX-2 passage number was restricted to between ten and twenty passages. 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 and seeded in either 2D or 3D environments in an appropriate manner for the experimental endpoint under investigation.

2.2.2 2D monolayer culture

For 2D monolayers, 20,000 HepG2 cells were seeded on 8-well culture slides (Corning, Thermo Fisher, Ireland). Cells were left to adhere for 24 h before use and cultured up to 7 d.

Supernatants were harvested at d 3, new medium added, and supernatants harvested again at d 7 of culture. Supernatants were stored at -20 °C before further analysis.

2.2.3 3D Spheroid formation

For HepG2 spheroid formation, cells were seeded using two different methods (Figure 2.1), the InSphero™ hanging drop system (InSphero™ AG, Sweden) and CellStar® 96 well round bottom cell repellent plates (Gibco, Invitrogen Ltd, VWR). These plates were chosen as they assist the formation of spheroids without the need for special supplements or culture substrates, which have been shown to have marked influence on the behaviour of various cell types.

The protocol provided by the manufacturer supplemented with information gathered from the scientific literature [299-301], was used to form the hepatic spheroids by the InSphero™ hanging drop system. Cells were seeded at varying densities (500, 1,000 and 2,000 cells per 40 µl of culture medium, the volume of a drop). Plates were then incubated for 3 d, allowing cells to loosely aggregated to form a spheroid. Spheroids were then transferred to GravityTrap™ Ultra-low adherence (ULA) plate as per manufacturer's protocol for prolonged culture. Media was changed every 2-4 d, and supernatants collected at the desired timepoints and stored at -20 °C for future analysis. 3D HepG2 spheroids were grown for up to 28 d.

In the CellStar® cell repellent plates, HepG2 cells were seeded in the plate wells, using densities of 1,000, 5,000 and 10,000 HepG2 cells per 100 µl culture medium, thus obtaining HepG2 spheroids. When forming multicellular hepatic spheroids, the LX-2 cells were pre-mixed with HepG2 cells in medium and the cell mixture seeded in CellStar™ cell repellent plates, to form the 3D multicellular spheroid model (Figure 2.3). In order to mimic the ratio between hepatocytes and hepatic stellate cells in the human liver, a protocol detailed in a recent publication from Pingitore *et al.* was followed [302]. 1:1, 5:1 and 24:1 were used as seeding ratios of HepG2 vs LX-2 cells. Spheroids of LX-2 cells alone were also formed and used as experimental control. 3D spheroids formed in CellStar® cell repellent plates were cultured for up to 32 d. Culture medium was changed every 3 d and harvested supernatants stored at -20 °C for future analysis.

For both methods, wells on the outer edges of the plate were not used, to avoid the edge effect, i.e. the greater susceptibility of wells at the plate edges to evaporation-induced effects [303, 304].

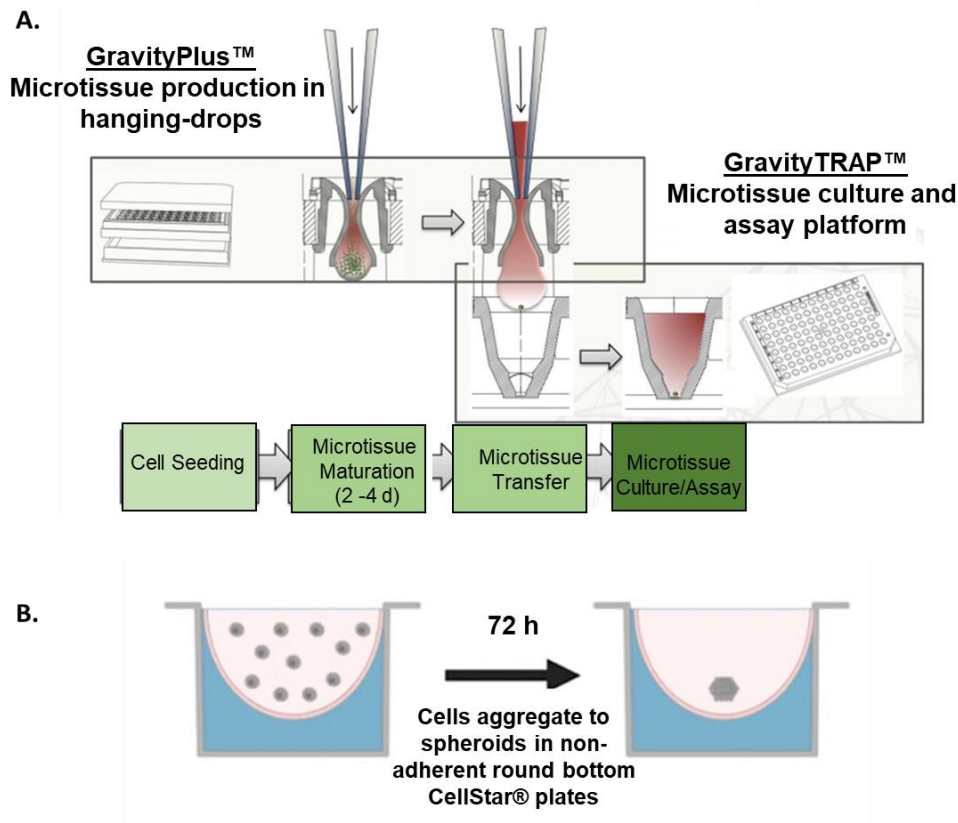


Figure 2.1. Hepatic spheroid formation by (A) InSphero™ hanging drop system and (B) CellStar™ cell repellent plates.

(A) Reproduced from InSphero™ website (insphero.com/science/).

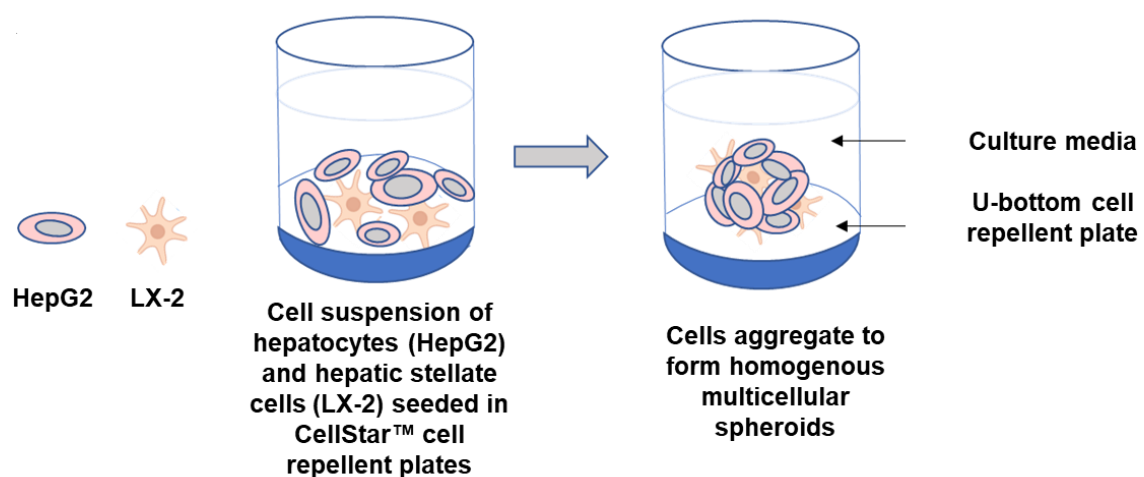


Figure 2.2. Illustration of multicellular hepatic spheroid formation using CellStar™ cell repellent plates.

2.2.4 Quantification of cell number in spheroids

Total cell number in hepatic spheroids was determined by adding TrypLE™ to spheroids (3 pooled spheroids), staining with 1:1 trypan-blue, and counting cells using the Countess™ automated cell counter.

2.2.5 Brightfield microscopy

Brightfield microscopy was used to assess the growth, morphology and size of hepatic spheroids at various time-points. Spheroids grown in InSphero plates, , were imaged by EVOS microscope (Thermo Fisher, Ireland) equipped with 10X objective every 4 d. Spheroids grown in CellStar® cell repellent plate, were imaged every 3 d by means of an epifluorescent microscope (Nikon TE300, equipped with 10X objective and QCapture Software QImaging). All image processing and measurements were undertaken using ImageJ software. For spheroid size measurements, the widest section of spheroid was measured. The average $\pm$ standard error of mean (SEM) is reported ($n_{\text{measurements}} = 10$ spheroid). Circularity and roundness parameters were obtained using ImageJ shape descriptor plugin. Circularity and roundness were calculated using equations 2.1 and 2.2 respectively:

$$\text{circularity} = 4\pi \times \left(\frac{\text{area}}{\text{perimeter}} \right)^2 \quad \text{Equation 2.1}$$

A circularity value of 1.0 is considered a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon.

$$\text{Round (roundness)} = 4 \times \text{area} / \pi \times \text{major axis}^2 \quad \text{Equation 2.2}$$

, or inverse of aspect ratio.

2.2.6 Histological analysis

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% agarose (low electroendosmosis, EEO) in PBS in plastic moulds. Cryopreservation of agarose blocks was first performed; however, cryopreservation was found to damage the structure of spheroids (please refer to Supplementary Figure S2.2). As a solution, 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 with the assistance of Anna Bogdanska (Nanomedicine and Molecular Imaging Group, Trinity College Dublin). Sections thus obtained were stained with haematoxylin and eosin (H&E). Briefly, slides with sectioned spheroids 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.

2.2.7 Immunofluorescent staining

Immunostaining was performed to analyse the formation of bile canalicular-like secondary structures. At the desired time-point, cell models were washed with pre-warmed PBS. After fixation with 4% PFA for 10 min at room temperature, specimens were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked in 1% BSA in PBS overnight. Cells/spheroids were then stained with antibodies as follows. Primary antibody (MRP2) (Thermo Fisher, Ireland) was diluted in 0.1% Triton X-100 in PBS at a concentration of 1:1000 and staining undertaken overnight at 4 °C, before washing and incubation with Alexa Fluor 568 goat anti-mouse secondary antibody (1:500). Nuclei and actin were stained using Hoechst 33342 (1:1000) and Alexa Fluor 488®-phalloidin (1:50) (both Invitrogen, Fisher Scientific, Ireland). Quantitative Laser Scanning Confocal Microscopy (LSCM) imaging was performed using a Leica SP8 confocal microscope equipped with a 20x dry objective. Z-stack images were acquired (49 steps, physical length 23.93 µm). Image processing undertaken using LAS X software.

2.2.8 Albumin quantification

Release of human serum albumin was quantified using R&D DuoSet Human Serum Albumin ELISA kit (R&D Systems, Ireland), carried out according to manufacturer's protocol on supernatants collected from cell models. Albumin content in supernatants was extrapolated and quantified from albumin standard curve included in the experimental design.

2.2.9 MRP2 transporter functionality

Cell cultures were incubated with 5 μ M CMFDA in the presence and absence of 5 μ M MK571 (an inhibitor of MRP2), for 30 mins at 37 °C. CMFDA is a membrane permeable compound which passively enters cells. Once inside cells, however, it is converted to GSMF (glutathione-methyl fluorescein), a cell impermeable compound which can only be removed from cells via transport through functional MRP2 transporters. After treatments, cultures were washed in pre-warmed PBS and stained with Hoechst (nuclei – 1:1000) and rhodamine phalloidin (F-actin – 1:40), both diluted in 5% BSA in PBS, for 1 h at room temperature (RT). Corrected total cell fluorescence (CTCF) was calculated for spheroids treated with CMFDA with and without MK571 inhibitor. Using ImageJ, five areas in representative images were highlighted and both fluorescence and background calculated. CTCF was calculated using the following equation (Equation 2.3).

$$CTCF = Integrated\ Density - (Area_{cell} \times Mean\ fluorescence_{Background}) \quad \textbf{Equation 2.3}$$

2.2.10 Viability assessment

2.2.10.1 CellTiter-Glo® luminescent 3D viability assay

Spheroid viability was assessed at 3, 7, 11, 15, 19, 23, 27 and 31 d, using the CellTiter-Glo® luminescent cell viability assay (Promega, MyBio, Ireland) following manufacture's protocol. This assay works by permeabilizing 3D cell structures and determining number of viable cells present via quantification of ATP content. Measuring intracellular ATP content is a powerful method of determining health, growth and energy status of cells with high confidence [305]. CellTiter-Glo® reagent is optimized for spheroids up to 900 μ m in size. After removing supernatants, cultures were washed with pre-warmed PBS, and 75 μ l of CellTiter-Glo® reagent was added to each well. Cultures were incubated on a shaker at RT in the dark for 30 min. After addition of CellTiter-Glo® reagent, spheroids may retain their

compact structure, however membrane will still be permeabilized enough to release ATP. Luminescence readings were carried out using the FLx800 microplate reader (Biotek, Mason Technologies, Ireland).

2.2.10.2 Live/dead assay

Ratio of live to dead cells in cultures was visually determined using a live/dead viability/cytotoxicity kit (Thermo Fisher, Ireland), as previously detailed in [306]. Briefly, 20 µl of 2 mM EthD-1 was added to 10 ml sterile PBS to obtain a 4 µM solution of EthD-1. 1. 5 µl of the supplied 4 mM calcein AM stock solution was then transferred to the EthD-1 working solution. The resulting solution was vortexed to ensure thorough mixing. After removal of medium, cultures grown for 4, 8, 12, 16, and 20 d were washed with pre-warmed PBS and incubated with the live/dead solution for 30 min at 37 °C. Cultures were then washed with PBS before being imaged by LSCM using a ZEISS 510 Meta confocal microscope equipped with a Zen imaging software (Carl Zeiss, Axiovert, Germany).

2.2.11 Drug Penetration

Following a protocol detailed in Gaskell *et al* [307], HepG2 spheroids were treated with 3 µg/ml of the autofluorescent compound doxorubicin for 24 h, before being washed with PBS, fixed with 4% PFA for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Spheroids were then incubated with Hoechst diluted in 0.1% Triton X-100 BSA for 1 h, before being imaged by LSCM, performed using a Leica SP8 confocal microscope equipped with a 20x dry objective. Image processing undertaken using LAS X software.

2.2.12 Sensitivity to hepatotoxins

Sensitivity of cell cultures to a panel of common hepatotoxins was tested using a protocol detailed previously in works from Gaskell *et al.* [307] and Bell *et al.* [299], and using information listed in the MIP-DILI training set [308]. The hepatotoxic compounds included: acetaminophen, diclofenac, trovafloxacin and fialuridine. HepG2 spheroids grown for 3 and 10 d were exposed to the hepatotoxic compounds diluted in cell culture medium, with an addition dose administered 2 d 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, as described in section 2.2.10. Cell culture media alone with Cell Titre-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). Three replicates were used for each experiment (n=3). A dose-response curve was plotted and IC50 values determined from the curve.

2.2.13 Statistical Analysis

All conditions were tested in triplicate ($n_{\text{replicates}} = 3$), and three independent experiments were carried out ($n_{\text{tests}} = 3$). Data are presented as average $\pm$ standard error of the mean. All graphs and statistical analysis were performed using GraphPad Prism 7. The statistical test carried out for each dataset is specified in the figure captions. $p < 0.05$ was considered statistically significant.

2.4 Results

The workflow for Chapter 2 is described in Figure 2.3.

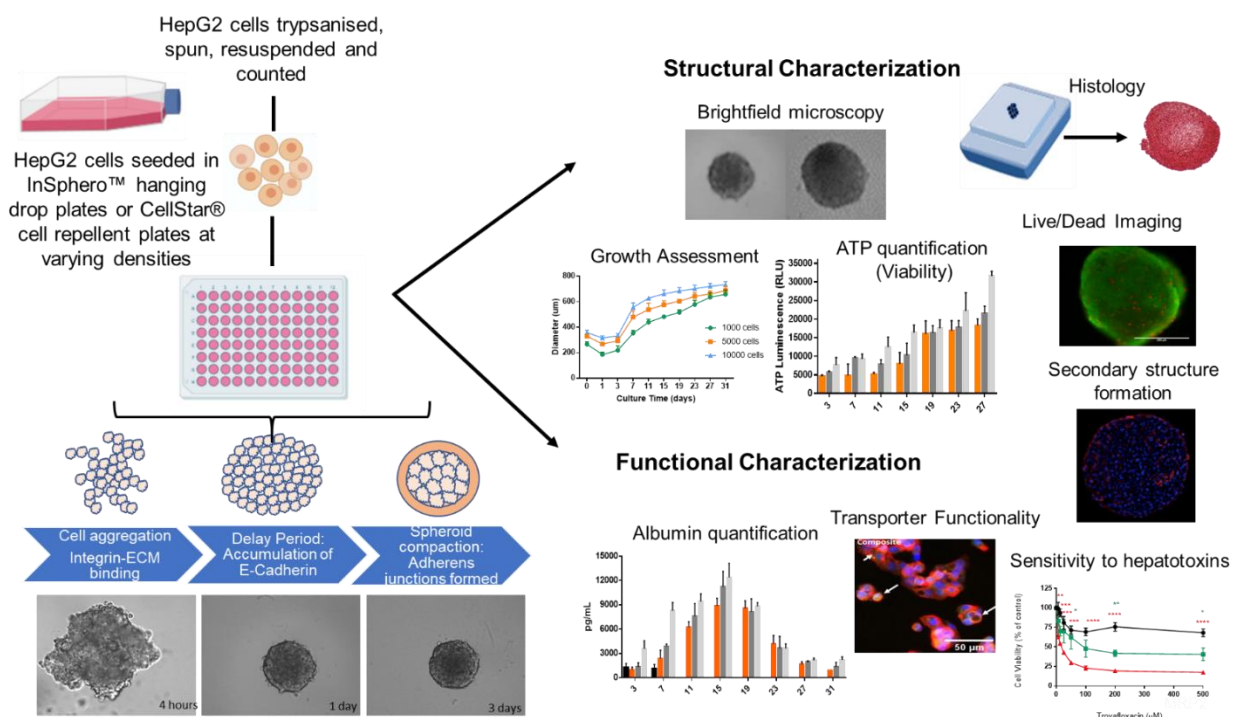


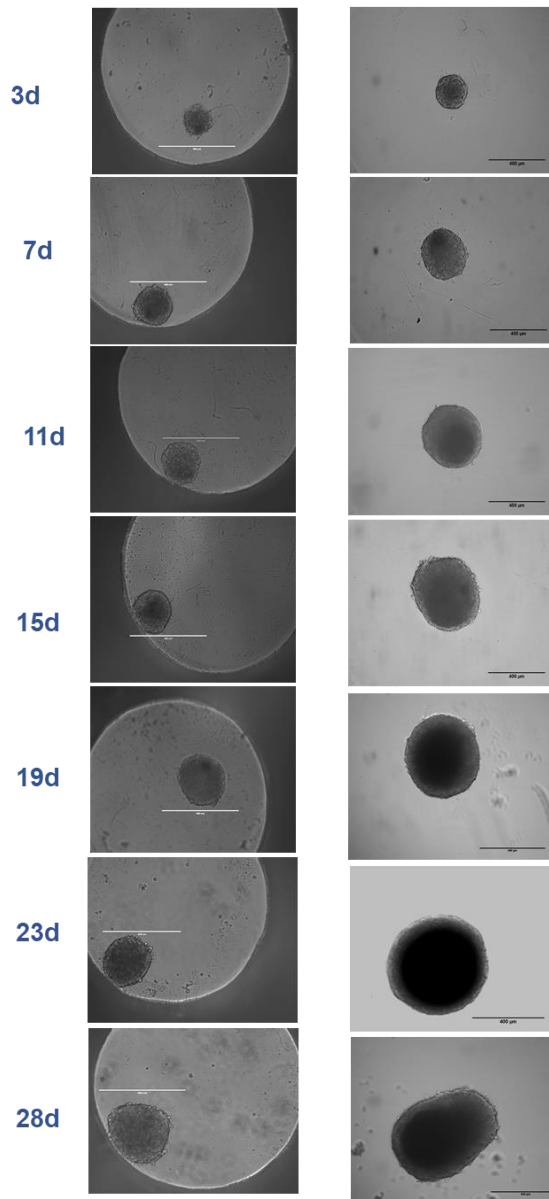
Figure 2.3. Workflow for the formation and characterization of HepG2 spheroids.

3D hepatic spheroids were formed using two commercial culturing plates, the InSphero™ hanging drop system and the CellStar® cell repellent plates, and then characterized for hepatic structure and function. Structural endpoints included morphology and growth assessment using brightfield microscopy, and histology for assessing spheroid internal viability and structure. Functional endpoints included albumin quantification via ELISA, ability to predict hepatotoxicity and secondary structure assessment using immunocytochemistry and confocal microscopy. Live/dead staining and ATP quantification were also used as a mean of determine spheroid viability over time.

2.4.1 Assessment of the most suitable methodology of forming 3D spheroids of HepG2 cells

Two commercial culture plates were used to form the hepatic spheroids: (i) the InSphero™ hanging drop system and (ii) CellStar® cell repellent plates. HepG2 cells aggregated to form one single spheroid per well using both these methods (Figure 2.4 A). Spheroid diameter gradually increased over the culture period, with similar growth patterns observed in both methods (Figure 2.4 B). However, there were distinct differences seen with spheroids formed using the two different methods. Spheroids created in CellStar® cell repellent plates were generally spherical and uniform in shape (Figure 2.4 A). In contrast, spheroids formed using the InSphero™ system were irregular in shape and had undefined perimeters. Spheroids formed by the InSphero™ system were smaller than those cultured on the CellStar® cell repellent plates and, in some cases, spheroid loss occurred during culture transfer from the drop to the companion plates. When cultured using the InSphero™ system, HepG2 spheroids tended to sit at the well edges, making their imaging and handling more difficult.

A. **InSphero™** **CellStar®**
hanging drop system cell repellent plates



B.

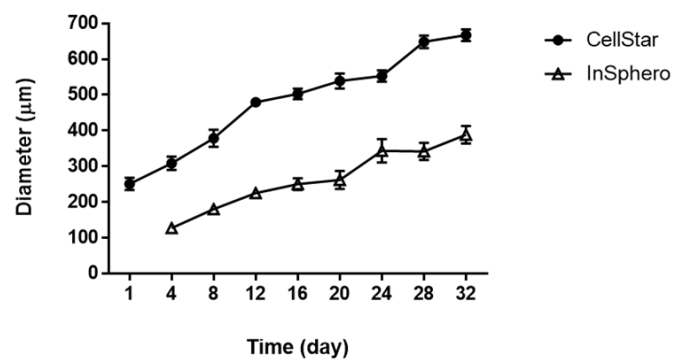


Figure 2.4. Comparison of hepatic spheroid formation using two different culturing plates.

(A) Brightfield images of hepatic spheroids formed seeding 1,000 HepG2 cells in CellStar™ cell repellent plates and using the InSphero™ system. Scale bar = 400 µm (B) Changes in the diameter (µm) of spheroids grown using both types of culture plates. Data are represented as mean ± SEM ($n_{\text{replicates}} = 6$).

The effect that cell seeding concentration has on spheroid formation, size and morphology was also assessed. In CellStar® round bottom plates (Figure 2.5), single, uniform spheroids were formed at each cell density tested (Figure 2.5 A). 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, originated the most uniform, circular spheroids across the seeding concentrations tested, with consistent morphology overtime and a diameter of 294.26 ± 10.43 µm at 3 d and of 647.95 ± 16.86 µm at 31 d. Spheroids formed from a starting density of 5,000 cells per 100 µl culture medium also increased in diameter steadily from 402.16 ± 11.82 µm at 3 d to 687.66 ± 28.79 µm at 31 d. Spheroids formed from a starting density of 10,000 cells per 100 µl culture medium produced the least consistent spheroids, which increased in size rapidly from 453.21 ± 15.36 µm at 3 d to 733.38 ± 22.36 µm at 31 d, but showing an irregular, blebbing shape (Figure 2.5 A). Following 31 d in culture, spheroid size was comparable among the different seeding concentrations (Figure 2.5 B). Similar experiments were carried out for InSphero™ plates. The results are reported in Appendix as these plates were not brought forward in the project due to the poor reproducibility evidenced by the protocol developed (please refer to Supplementary Figure S2.1).

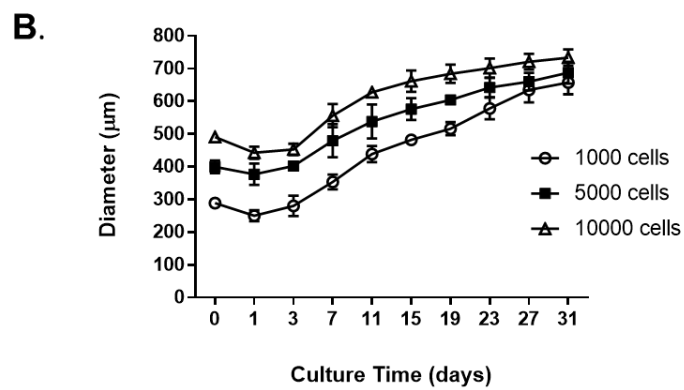
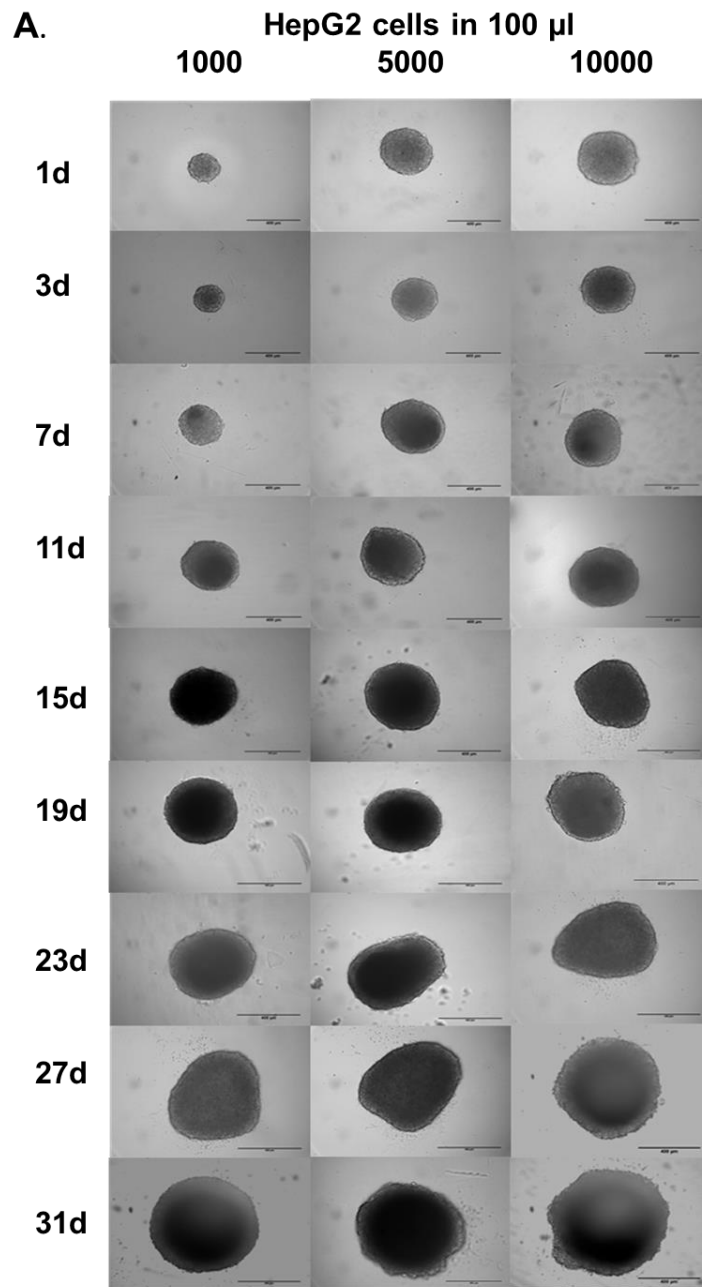


Figure 2.5. Effect of cell seeding concentration on HepG2 spheroid formation.

(A) Representative brightfield microscopy images of HepG2 spheroids formed by CellStar® cell repellent plates starting from cell seeding concentrations of 1,000, 5,000 and 10,000 cells per 100 μ l culture medium. Scale bar = 400 μ m. (B) Changes in spheroid diameter (μ m) over 31 d in culture. Data are represented as mean $\pm$ SEM ($n_{\text{replicates}} = 10$).

The increase in spheroid diameter was reflected in an increase of cell number overtime (Figure 2.6). 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.

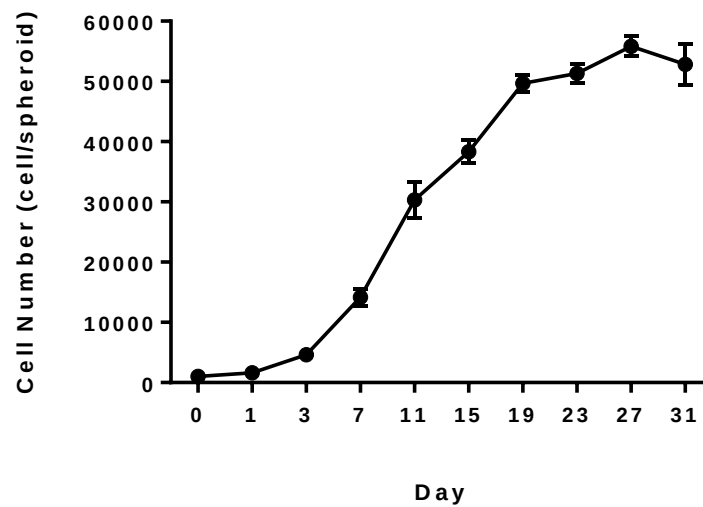


Figure 2.6. Cell number in HepG2 spheroids grown in CellStar® cell repellent plates.

Spheroids were formed starting from a seeding concentration of 1,000 HepG2 cells per 100 μ l culture medium and cultured for 31 d. Data are shown as mean $\pm$ SEM ($n_{\text{replicates}} = 6$).

The roundness and circularity of the HepG2 spheroids were then analysed determined using ImageJ shape descriptors (Figure 2.7). At each cell density, little deviation was observed in both circularity or roundness.

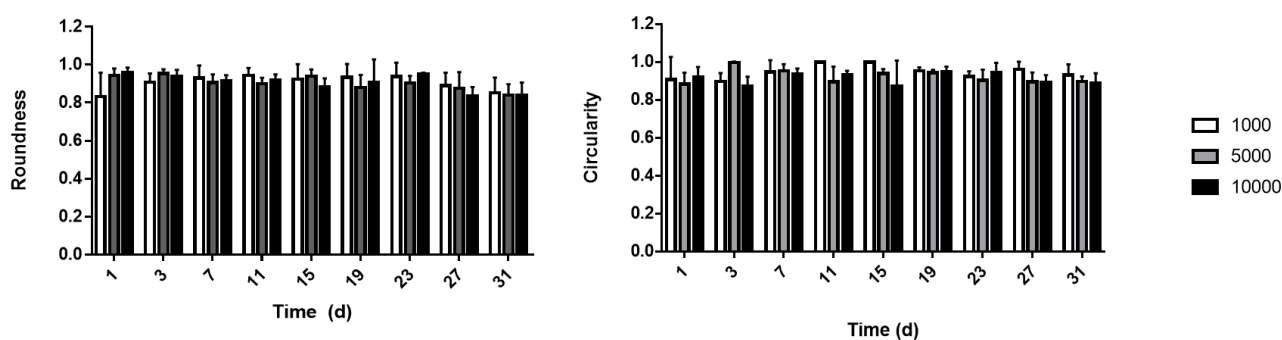


Figure 2.7. Changes in roundness and circularity of HepG2 spheroids formed in CellStar® cell repellent plates.

Spheroids were formed starting from cell seeding concentrations of 1,000 (white bars), 5,000 (grey bars) and 10,000 (black bars) cells per 100 µl cell culture medium. Spheroids were cultured for 31 d and imaged using brightfield microscopy. Spheroid diameter was calculated by ImageJ, using an image of known scale for calibration. Spheroid circularity and roundness were measured using image descriptors built into ImageJ software. Data are represented as mean $\pm$ SEM ($n_{\text{replicates}} = 10$).

2.4.2 Structural and Functional Characterization of model

2.4.2.1 Spheroid Viability

Intra-micro tissue ATP content (indicative of cell viability) was assessed using the CellTiter-Glo® 3D luminescent viability assay over 31 d, to determine if cells in the spheroid remained viable as spheroids grew in size. This ready-to-use assay was chosen as it facilitates the time-effective and standardized processing of large spheroid numbers at the same time by combining cell lysis and luminescence signal formation in a single step. Spheroids remained viable throughout the culture period with a density-dependent increase in luminescence observed until 19 d or 23 d, when the ATP content started to decrease reaching its minimum at 31 d (Figure 2.8).

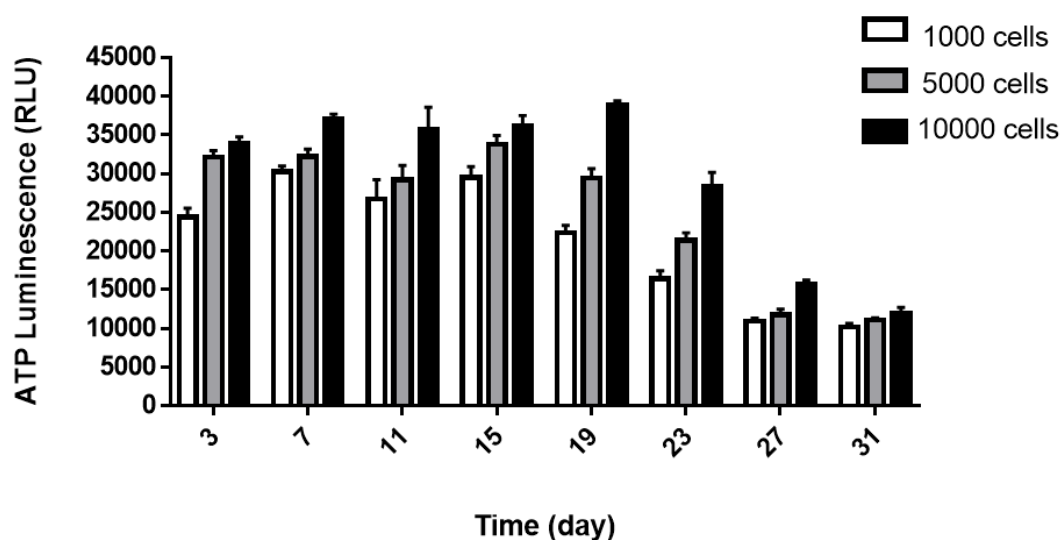


Figure 2.8. Viability of HepG2 spheroids overtime.

The viability of HepG2 spheroids grown from 1,000 (white bars), 5,000 (grey bars) and 10,000 (black bars) cells per 100 μ l cell culture medium was determined using the CellTiter-Glo[®] luminescent viability assay over a 31d culture period. Spheroids remained viable throughout this time period, with luminescence increasing with increasing densities until 19 d (for spheroids grown from 1,000 and 5,000 cells per 100 μ l cell culture medium) or 23 d (for spheroids grown from 10,000 cells per 100 μ l cell culture medium). Data are represented as mean $\pm$ SEM ($n_{\text{replicates}} = 9$).

2.4.2.2 Internal spheroid health and structure

In order to assess changes in internal spheroid structure and in morphology over 31 d in culture, HepG2 spheroids were paraffin-embedded, sectioned and stained with H&E. Spheroids formed from a cell seeding concentration of 1,000 cells per 100 μ l culture medium, confirmed a compact, uniform internal structure with defined outer perimeters (Figure 2.9), and direct cell-cell contacts similar to those observed in human liver (See Supplementary Figure 2.4). Spheroids size increased overtime (Figure 2.9), confirming results obtained by brightfield microscopy (Figure 2.5). Limited apoptosis and no necrotic core was visible up to 27 d, when a small patch of necrosis could be observed in spheroid core. In contrast, spheroids grown from a seeding concentration of 5,000 cells per 100 μ l culture medium presented changes in morphology already at day 11, and noticeable patches

of cell death appearing after 15 d. Spherical morphology was completely lost at 27 d and 31 d.

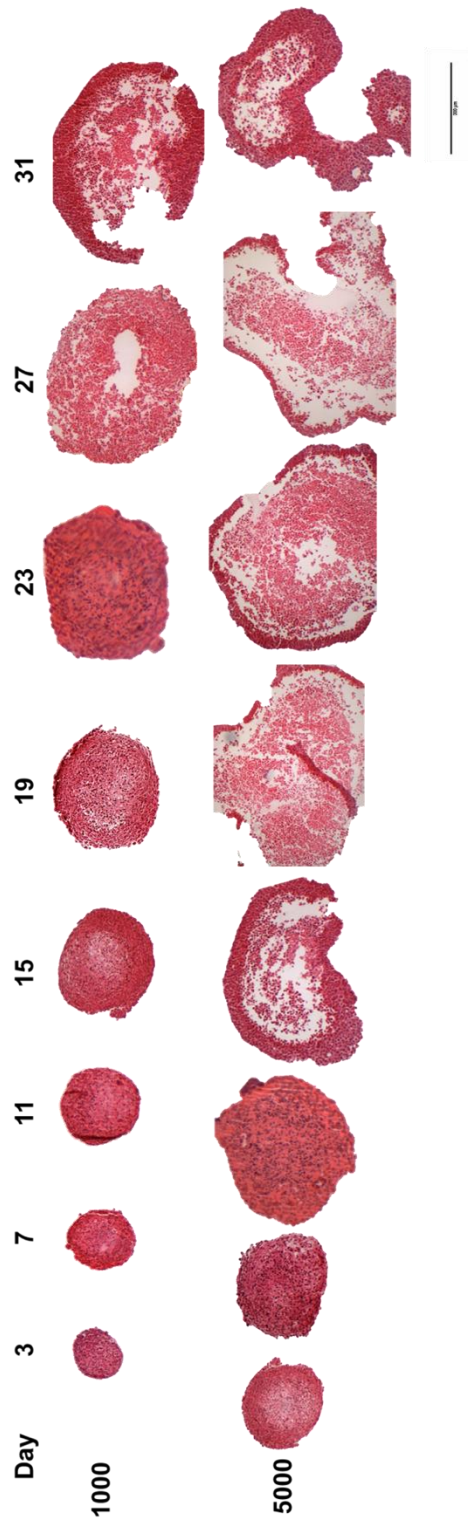


Figure 2.9. Internal spheroid structure and morphology.

Spheroids were created using CellStar® cell repellent plates from 1000 and 5000 HepG2 cells in 100 µl cell culture medium. Spheroids were fixed in PFA after 3, 7, 11, 15, 19, 23, 27 and 31 d in culture, before being suspended in agarose, dehydrated in increasing concentrations of ethanol, paraffin embedded, sectioned and stained with H&E. Images are representative of the specimens and show mid-section of the spheroids. Scale bar = 300 µm.

2.4.2.3 Polarisation of HepG2 cells in hepatic spheroids

Immunofluorescent analysis was carried out to evaluate potential changes in hepatocytes polarisation over time. Polarisation is a key feature of hepatocytes. The apical poles of adjoining hepatocytes join to form continuous channels, i.e., bile canaliculi, that contain many transport proteins including multi-drug resistance-associated protein 2, or MRP2, which excrete bile salts from cells [84, 309, 310]. HepG2 spheroid was stained with Hoechst (blue – nuclei) and Alexa Fluor 488-phalloidin (F-actin - green) to visualise actin structures by epifluorescence microscopy (Figure 2.10). After 11 d, actin structures were joined together, forming interconnected networks of secondary structures within the spheroids.

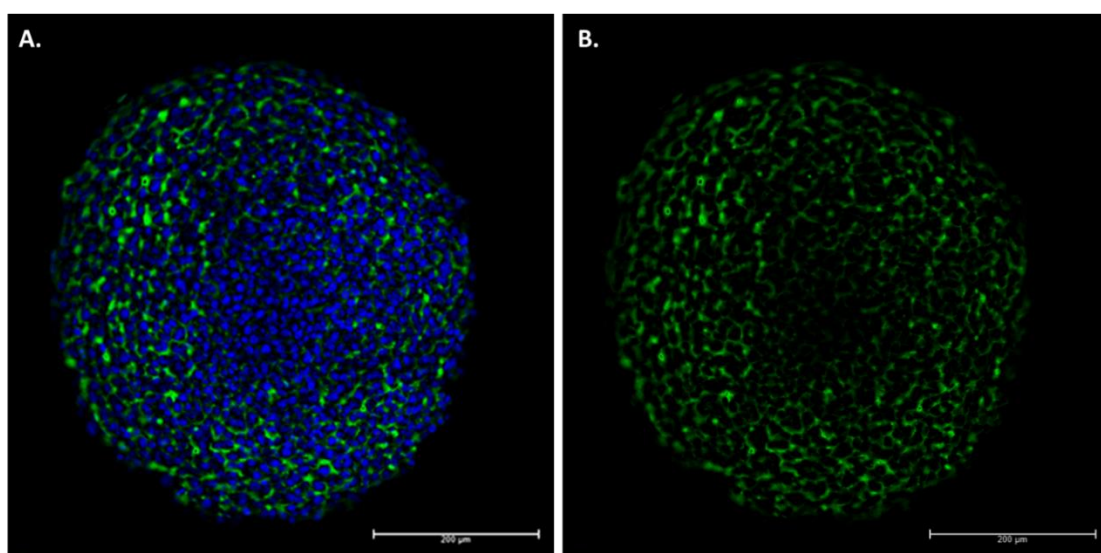


Figure 2.10. Formation of secondary structures in HepG2 spheroids.

HepG2 cells cultured for 11 d using cell repellent plates were fixed, permeabilized, and blocked before staining with Hoechst (blue) to stain the nuclei and Alexa Fluor 488-phalloidin (green) to stain F-actin. Image A represents nuclei and actin merge image, B represents actin alone. Image obtained using epifluorescence microscopy and 10x magnification. Scale bar = 200 µm.

Staining with an MRP2 antibody was undertaken to confirm that the interconnect actin structures seen in Figure 2.11 were a consequence of hepatic cell polarisation, with varying densities and time points used to determine if size or culture period affected the expression of MRP2. Thus, spheroids at various seeding densities (1,000, 5,000 and 10,000 cells per

100 µl culture medium) were analysed at 4, 11 and 18 d for MRP2 expression. Secondary structures begin to form after 4 d for all seeding densities (Figure 2.11 A and Supplementary Figure S2.3) and elongate and join further at 11 d and 18 d to form canalicular-like networks. The MRP-2 staining pattern observed overlapped with the secondary structures shown in Figure 2.10. Conversely, a similar staining pattern could not be detected in conventional 2D monolayers, grown and stained as control (Figure 2.11 B).

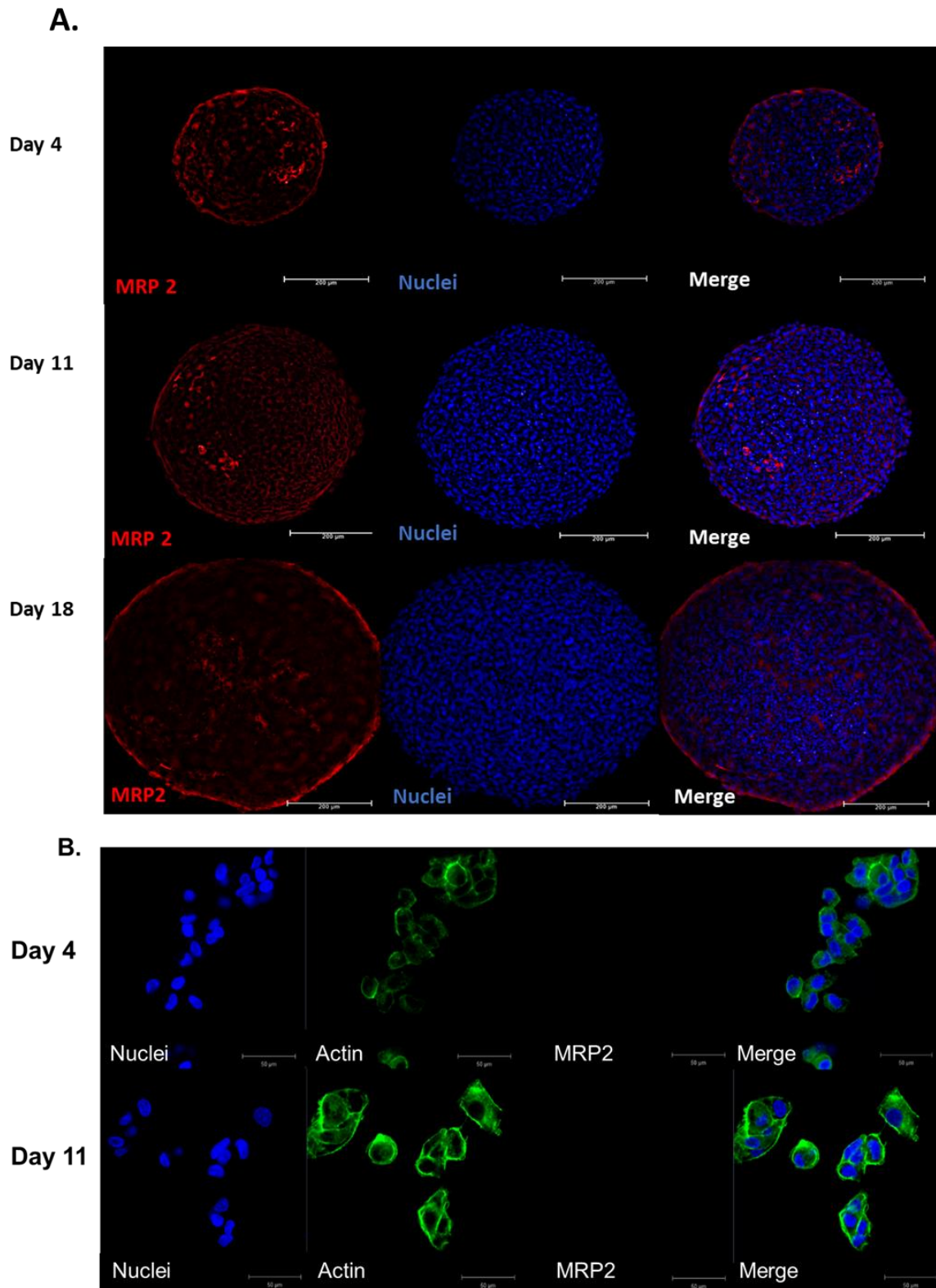


Figure 2.11. Expression of the MRP2 transporter in HepG2 cells cultured as (A) 3D spheroids or (B) 2D monolayers.

Representative LSCM images of: (A) HepG2 spheroids formed from a cell seeding concentration of 1,000 cells per 100 μ l culture medium and cultured for 4, 11 and 18 d; (B)

2D monolayer controls formed by seeding 10,000 per 100 μl culture medium and cultured for 4 and 11 d. (A-B) Specimens were stained with MRP2 antibody (red – canalicular transporter) and Hoechst (blue - nuclei). Scale bars = 200 μm (20x dry objective).

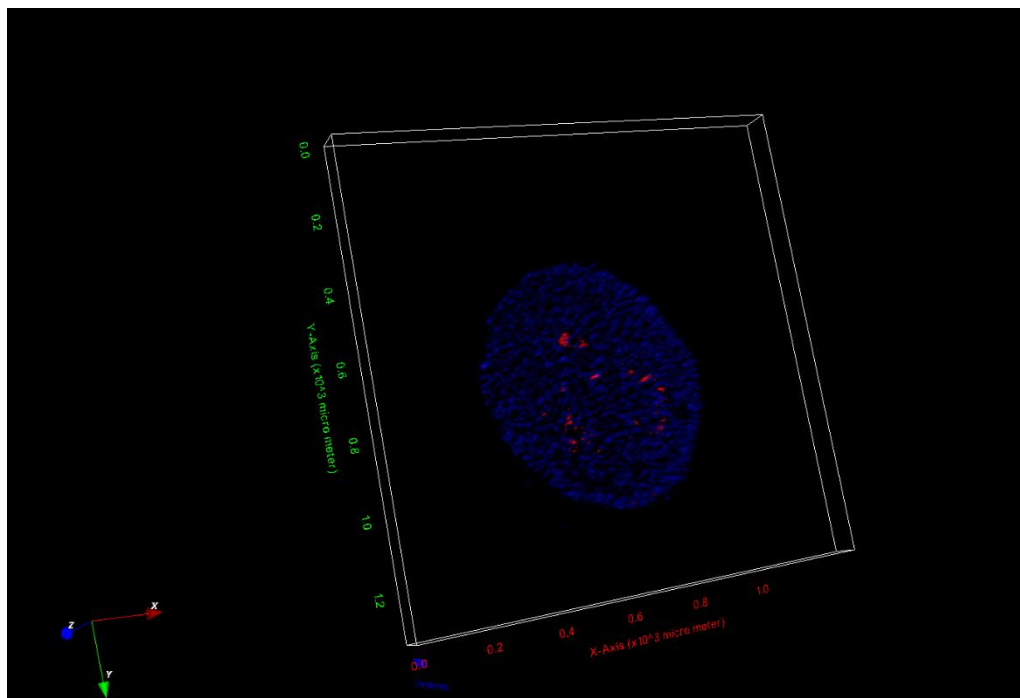


Figure 2.12. Representative images of segmented 3D volumes from HepG2 spheroid stained with MRP2 and imaged by LSCM.

Bile canaliculi-like structures denoted in red. Initial z-stack images (Z-stack 137 steps) acquired using LSCM were reconstructed into a virtual stack; each of the segmented classes was then assigned a pseudo-colour (MRP2 in red; nuclei in blue) for representative 3D volume. The Icy v (Version 2.0.3.0) software (open community platform for bioimage informatics, <http://icy.bioimageanalysis.org/>) with built in Visualization Toolkit (3D VTK) for 3D computer graphics, was used to generate the 3D volume.

2.4.2.4 Quantification of human serum albumin using ELISA

Albumin secretion is often used to assess liver-specific function in spheroids [307, 311-313]. The production of albumin was therefore quantified over 31 d, on supernatants collected from spheroids. Albumin secretion increased over the culture period up to its maximum cumulative release at 15 d (Figure 2.10), before dropping after 19 d in culture. After 3 d, the albumin secretion of 3D spheroids was comparable to that of 2D monolayers after 3 d, with the exemption of 3D spheroids grown from a cell seeding concentration of 10,000 cells per 100 μ l culture medium. Conversely, it was more than doubled after 7 d in culture (Figure 2.13).

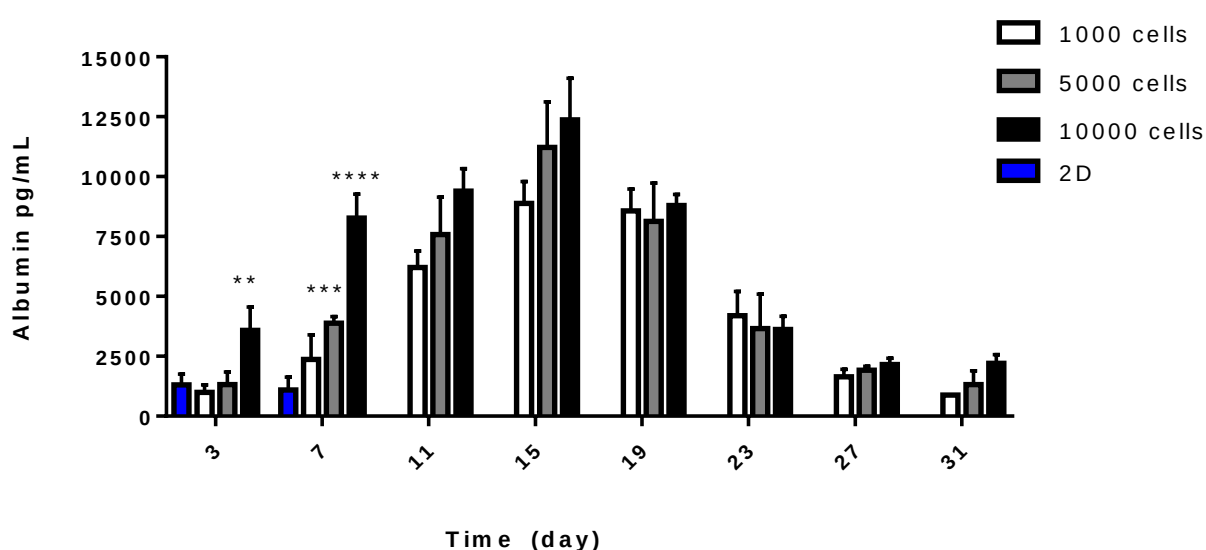


Figure 2.13. Changes in albumin release from HepG2 spheroids and 2D monolayers overtime.

Supernatants from 2D HepG2 cultures and 3D spheroids were collected and analysed over the course of 31 d. HepG2 spheroids were grown starting from a seeding concentration of 1,000 (white bars), 5,000 (grey bars) and 10,000 (black bars) cells per 100 μ l cell culture medium. 2D monolayers (blue bars) were formed by seeding 10,000 per 100 μ l culture medium. Data represented as mean $\pm$ SEM, Two-way ANOVA ** p 0.0030, *** p 0.0005, and **** p <0.0001 ($n_{\text{replicates}}$ = 3 in triplicate).

2.4.2.5 Confirmation of transporter functionality

Further experiments were carried out to determine if canalicular transporters were in fact also functionally active. Functionality of MRP2 was studied using CMFDA, a permeable fluorescent probe which enters living cells readily, where it is transformed into the fluorescent compound glutathione-methyl fluorescein (GSMF), which can only be expelled from cells via functional MRP2 transporters. Figure 2.14 A shows that, in 2D HepG2 monolayers, CMFDA was retained in the cytoplasm of most cells. Conversely, in 3D spheroids, a limited retention of CMFDA was seen in cell cytoplasm (Figure 2.14 B), with accumulation and co-localisation observed with secondary canalicular-like structures (indicated by white arrows). These data seem to suggest that CMFDA was actively transported from the cells by the MRP2 into the canalicular-like structures. Further supporting this, in the presence of MK571, as an MRP2 transporter inhibitor. CMFDA accumulation in the HepG2 cell cytoplasm increased in 3D spheroids (Figure 2.14 C). Based on my quantification, the corrected total cell fluorescence (CTCF) for CMFDA-treated spheroids was 33,841 a.u.; whereas, in the presence of the MRP2 inhibitor, the CTCF was equal to 103,064 a.u. (Figure 2.14 D).

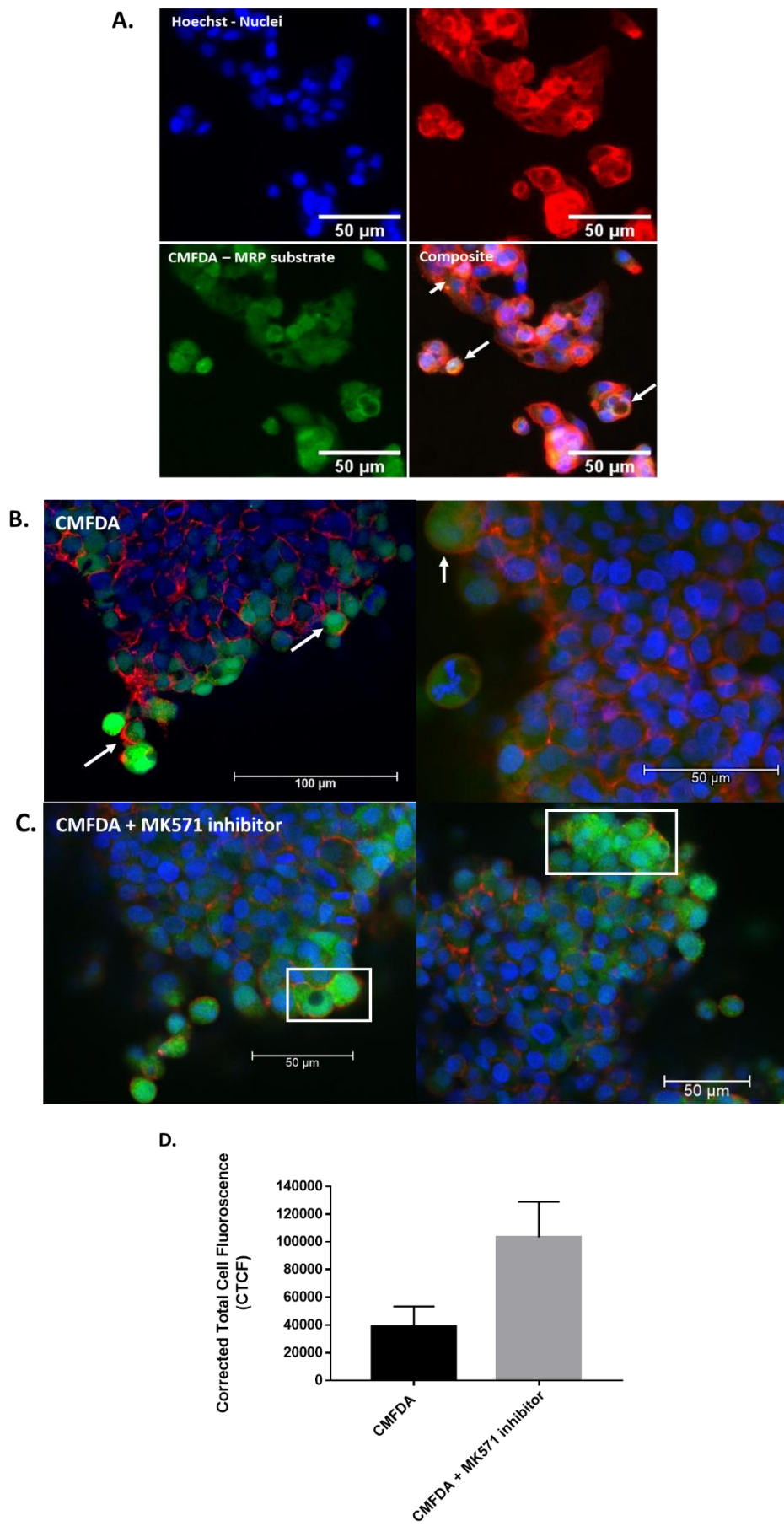


Figure 2.14. Transporter functionality in HepG2 spheroids.

(A) HepG2 monolayers and (B) HepG2 spheroids were cultured for 8 d before being incubated with CMFDA, an MRP2 transporter substrate (green) for 30 min. (C) HepG2 spheroids incubated with CMFDA and 25 μ M MK571, an MRP2 inhibitor. Monolayers and spheroids were washed, fixed, permeabilised and stained with Hoechst (nuclei – blue, 1:1000) and rhodamine phalloidin (actin – red, 1:40) for 1 h at RT in the dark. (A) imaged using Lionheart FX automated microscope (Biotek, Ireland). Scale = 50 μ m. (B) imaged using a Leica SP8 confocal microscope equipped with a 20x dry objective. Image processing undertaken using LAS X software. Scale bars = (A) 100 μ m and (B) zoomed image 50 μ m. (A) Arrows indicate regions where CMFDA is retained in the cell cytoplasm. (B) Arrows indicate regions where canalicular-like structures contain CMFDA. (C) White boxes demote examples of areas where CMFDA is retained in the cytoplasm, indicative of transporter inhibition. LSCM images undertaken with the assistance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, Trinity College Dublin, using a Leica SP8 confocal microscope equipped with a 20x dry objective. Image processing undertaken using LAS X software. Scale bars = (A) 100 μ m and (B) zoomed image 50 μ m. (D) Fluorescence intensity in HepG2 spheroids incubated with CMFDA with and without MK571. Analysis undertaken using ImageJ. Data represented as mean $\pm$ SEM.

2.4.2.6 Live/Dead imaging

The live/dead immunofluorescent assay was used to evaluate cell viability within spheroids over 20 d in culture (Figure 2.15). Up to 16 d, a small proportion of dead cells could be observed in spheroids. However, when spheroids were cultured beyond this time point, and as spheroids increased in size, a greater proportion of dead cells was observed. However, the live/dead fluorescent assay is significantly limited by the ability of the respective dyes to penetrate larger spheroids, hence spheroids were only tested for 20 d.

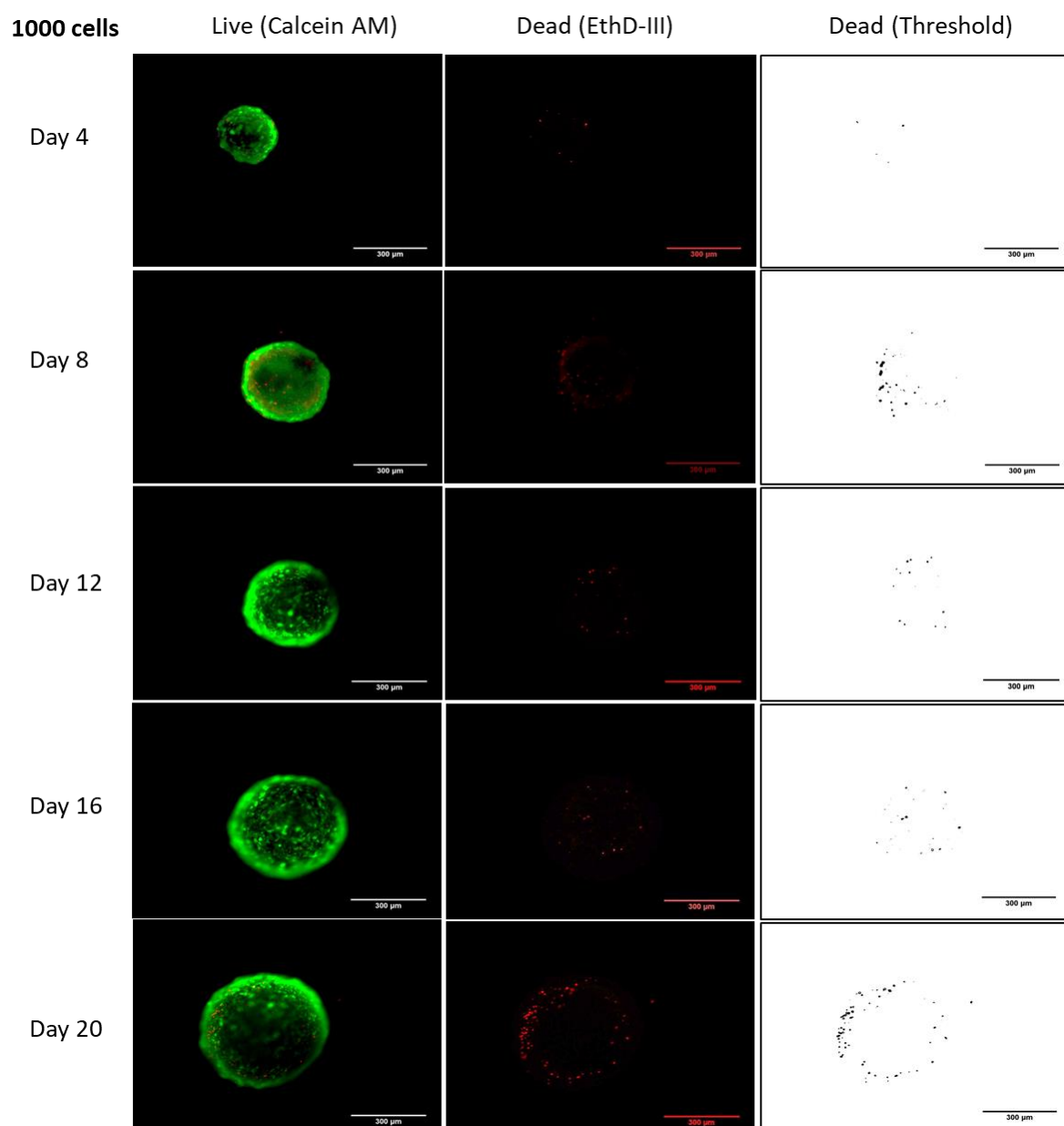


Figure 2.15. Representative images of HepG2 spheroids imaged by live/dead immunofluorescent assay.

Spheroids (cell seeding concentration: 1,000 cells per 100 μ l culture medium) were treated for 30 min with 1 μ M calcein AM (to stain live cells) and 4 μ M ethidium homodimer (to stain dead cells) before being imaged via LSCM using a Zeiss 510 Meta confocal microscope. Images processed using Zen software (Carl Zeiss, Axiovert, Germany). Images were processed and thresholded using ImageJ software. Scale bar = 300 μ m.

2.4.2.7 Drug penetration in spheroids

In order to determine if compounds could successfully penetrate spheroids homogeneously, spheroids were treated with doxorubicin. Doxorubicin was chosen as it is an auto-fluorescent compound. Spheroids cultured for 8 d and with a diameter of approximately 280 μm , were treated with doxorubicin for 24 h before being fixed, permeabilized, stained with Hoechst to visualize the nuclei, and imaged by LSCM. Z-stack images showed that doxorubicin could be detected throughout the spheroids, from the periphery to the middle core section. This indicates that doxorubicin is indeed likely to be in contact with all cells in the spheroid, and that drug response from compound is from all cells and not just a specific subsection (Figure 2.16).

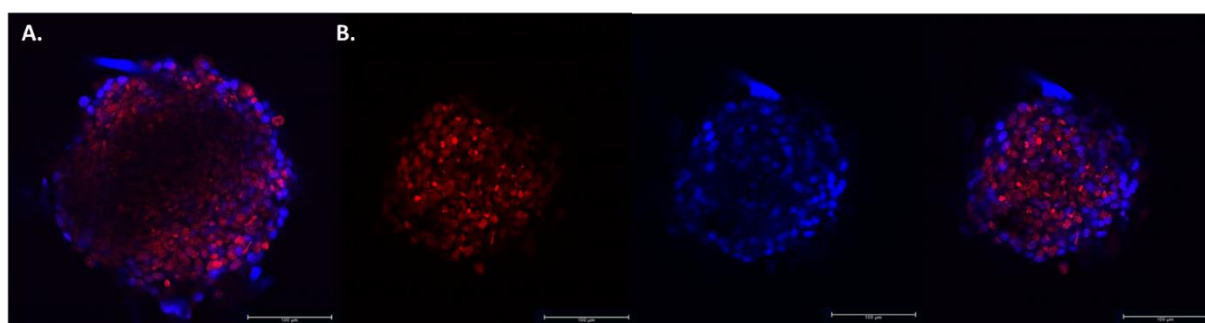


Figure 2.16. Doxorubicin penetration through 3D HepG2 spheroids

Representative LSCM images of HepG2 spheroids cultured using CellStar® cell repellent plate, starting from a cell seeding density of 1,000 cells per 100 μl culture medium, and treated with 3 $\mu\text{g/ml}$ doxorubicin (shown in red) for 24 h. Cell nuclei are stained with Hoechst (in blue). (A) Maximum intensity projection image; (B) Image of one section at the spheroid core. Quantitative Laser Scanning Confocal Microscopy (LSCM) imaging was performed using a Leica SP8 confocal microscope equipped with a 10x objective. Z-stack images were acquired (27 steps, physical length 13.73 μm). Image processing undertaken using LAS X software. Scale bar = 100 μm (10x objective).

2.4.3 Ability of spheroids to predict hepatotoxicity

2.4.3.1 Sensitivity of HepG2 spheroids to detect human hepatotoxins and comparison to 2D monolayers

Spheroids and HepG2 monolayers were treated with varying concentrations of four common hepatotoxic compounds namely, acetaminophen (an analgesic drug commonly used to treat fevers) diclofenac (a non-steroidal anti-inflammatory drug), fialuridine (a nucleoside analogue investigated for its potential as a hepatitis B virus infection therapy) and trovafloxacin, a broad spectrum antibiotic. Information on these compounds and how they impact the liver is listed below in the discussion of this chapter in Table 2.4. Following a protocol detailed in Gaskell et al [307], 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 2.17). Each of the five chosen compounds was significantly more toxic in 3D spheroids than in 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 2.1. A comparison to data extracted from the scientific literature is also reported.

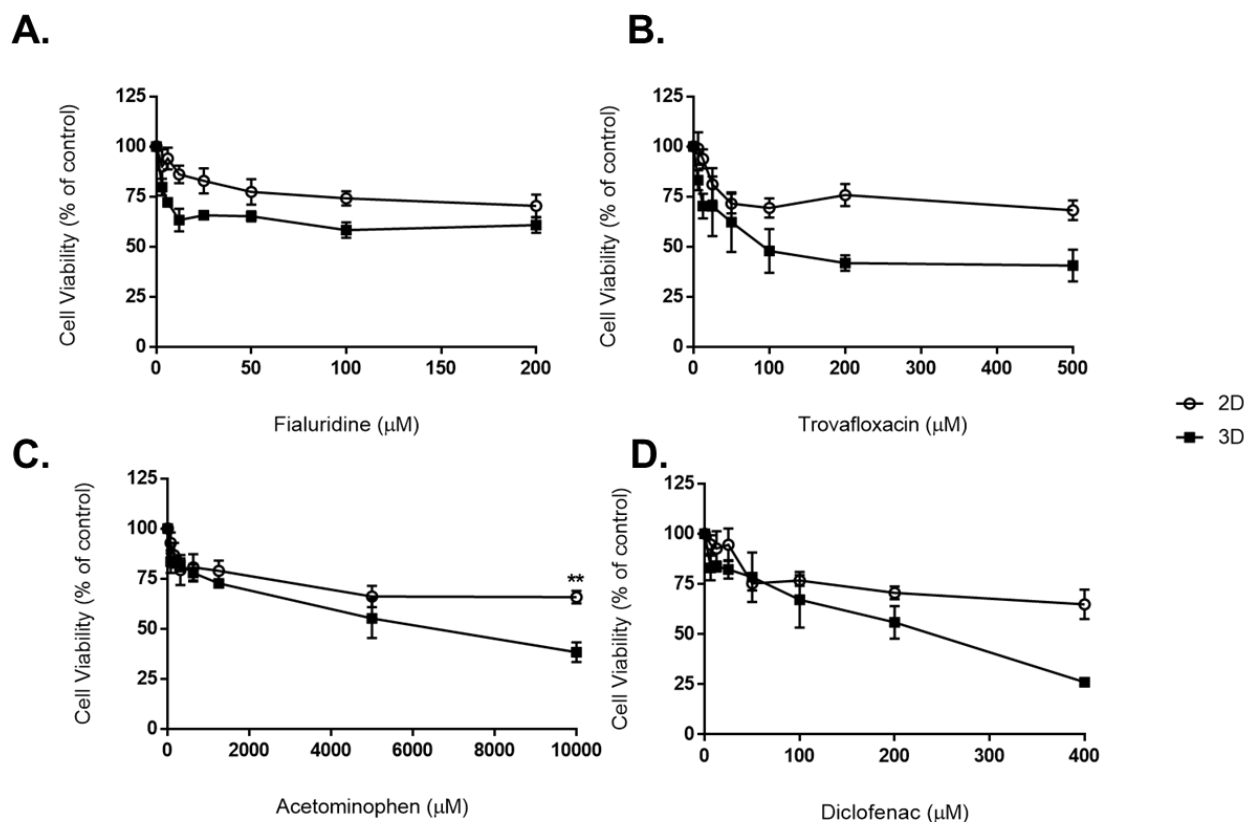


Figure 2.17. Changes in cell viability in 2D monolayers and 3D spheroids of HepG2 cells following exposure to four common hepatotoxins.

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).

Table 2.1. Sensitivity of HepG2 spheroids and monolayers to a panel of common hepatotoxins.

HepG2 cells and spheroids were treated with varying concentrations of common hepatotoxins. ATP was quantified to assess viability, and the concentration of each drug to cause a 50% reduction in viability was estimated from assay results, with comparisons made to appropriate literature. PHH denoted primary human hepatocytes.

Compound	Value at which 50% reduction in viability is observed (μM)			
	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
Trovaflaxacin	>100	>400	>1000	~200
Acetaminophen	>8000	>10000	22800	~20000
Diclofenac	>250	>400	763	331

In order to determine if the sizes of spheroids or their duration in culture affected response to hepatotoxins, the same experiment was repeated on spheroids cultured for 10 d, with repeated dosing undertaken at d12. As detailed in Figure 2.18, no significant difference in toxicological response was observed between spheroids treated with the chosen hepatotoxins at day 3 or day 10. Estimated values where each hepatotoxin would cause a 50% reduction of cell viability were comparable for all hepatotoxins, i.e. fialuridine: >200 μM (3 d) and >200 μM (10 d), Trovaflaxacin: >100 μM (3 d) and >200 μM (10 d), Acetaminophen: >8,000 μM (3 d) and >10,000 μM (10 d), Diclofenac: >250 μM (3 d) and >200 μM (10 d).

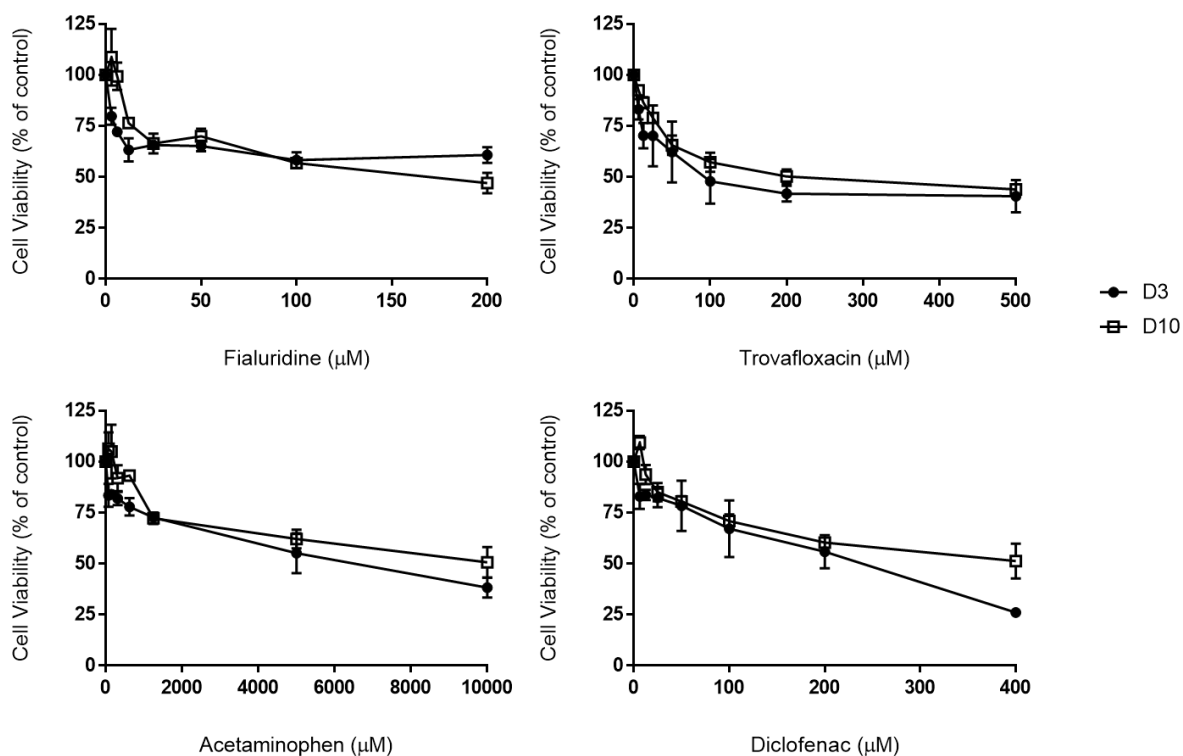


Figure 2.18. Effect of culture time on spheroid response to hepatotoxins.

Spheroids were cultured for both 3 d or 10 d and treated with the four chosen hepatotoxins (acetaminophen, diclofenac, fialuridine, and trovafloxacin) with repeated dosing at day 5 and day 12. Cell viability was determined using ATP quantification and results plotted as a percentage of untreated control. Data are represented as mean $\pm$ SEM ($n=3$ in duplicate). Changes were not statistically different (multiple t tests with Bonferroni Dunn method).

2.4.4 Development and characterization of a multicellular hepatic spheroid model

2.4.4.1 HepG2/LX-2 multicellular spheroids: growth, morphology and viability

Using a protocol similar to that developed for forming 3D spheroids from HepG2 cells, multicellular spheroids were formed successfully from a mixture of two hepatic cell types (HepG2 and LX-2 cell lines) in cell repellent plates (Figure 2.19). Spheroid diameter gradually increased over the culture period, from $265 \pm 20.65 \mu\text{m}$ at 24 h, to $305 \pm 8.73 \mu\text{m}$ at 72 h, and finally to $403.25 \pm 10.82 \mu\text{m}$ after 8 d (Figure 2.21). In comparison, spheroids of LX-2 cells alone formed looser spheroids for the first three days in culture (Figure 2.19), with diameters ranging from $391.25 \pm 16.72 \mu\text{m}$ at 24 h to $448.75 \pm 23.01 \mu\text{m}$ at 72 h (Figure 2.20). After 96 h, the LX-2 spheroids started to be more compact (Figure 2.19), with defined perimeter and diameter of $450 \pm 15.81 \mu\text{m}$, which increased in size to $510.5 \pm 29.41 \mu\text{m}$ after 8 d (Figure 2.20).

HepG2/LX-2 multicellular spheroids were formed at varying ratios (1:1, 5:1 and a physiologically relevant 24:1) and cell seeding densities (1,000, 2,000 and 5,000 cells per 100 μl culture medium). Spheroids formed successfully from all cell ratios and densities (Figure 2.19). Nevertheless, multicellular spheroids formed from the lowest cell density (1,000) produced the most spherical and consistent spheroids, with increased amounts of blebbing and less spherical morphology observed for 2,000 and 5,000 cell seeding densities (Figure 2.19). The LX-2 cells also profoundly impacted the morphology of the spheroids. Sizes ranged from $333.14 \pm 16.03 \mu\text{m}$ (1:1 ratio), $384.19 \pm 19.14 \mu\text{m}$ (5:1 ratio) and $401.26 \pm 14.3 \mu\text{m}$ (24:1 ratio) at 24 h, to $385.12 \pm 32.1 \mu\text{m}$ (1:1 ratio), $381.35 \pm 18.46 \mu\text{m}$ (1:5 ratio) and $415.03 \pm 29.10 \mu\text{m}$ (24:1 ratio) at 72 h. Multicellular spheroids reached sizes of $440.43 \pm 10.16 \mu\text{m}$ (1:1 ratio), $485.36 \pm 16.23 \mu\text{m}$ (1:5 ratio) and $515.41 \pm 17.21 \mu\text{m}$ (24:1 ratio) at 8 d (Figure 2.20). Spheroids also appeared most spherical at the most physiologically relevant ratio, 24:1 (Figure 2.19).

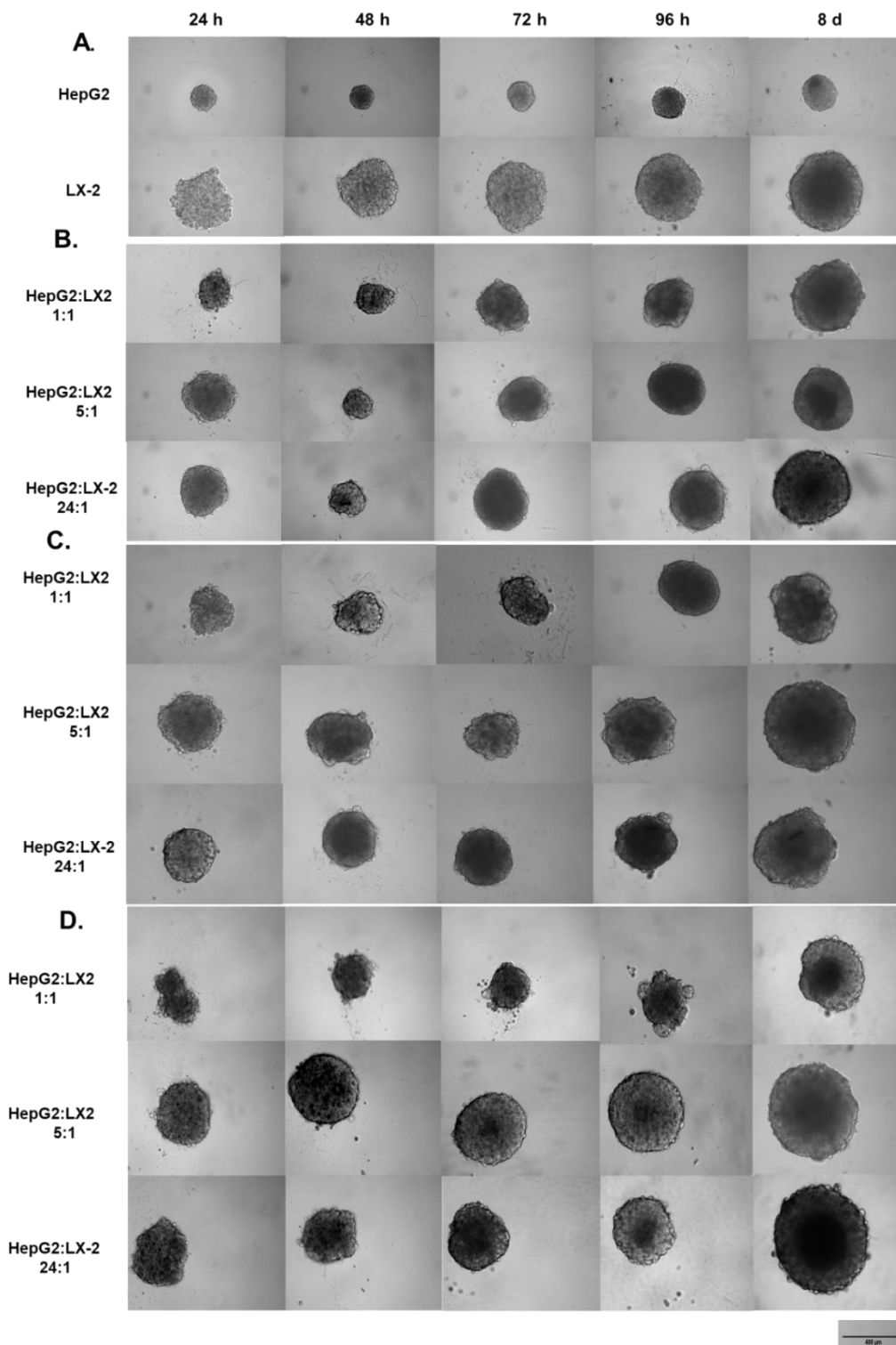


Figure 2.19. Formation of HepG2/LX-2 multicellular spheroids.

Representative brightfield microscopy images of (A) HepG2 and LX-2 monocellular spheroids, and (B-D) HepG2/LX-2 multicellular spheroids grown up to 8 d. (A-D) Spheroids were formed using CellStar® cell repellent plates. (A) 1000 cell seeding density per well used (B-D) Varying HepG2:LX-2 cell ratios (1:1, 5:1 and 24:1) and cell seeding densities were used: (B) 1,000, (C) 2,000, and (D) 5,000 cells per 100 μl culture medium. . Scale bar = 400 μm.

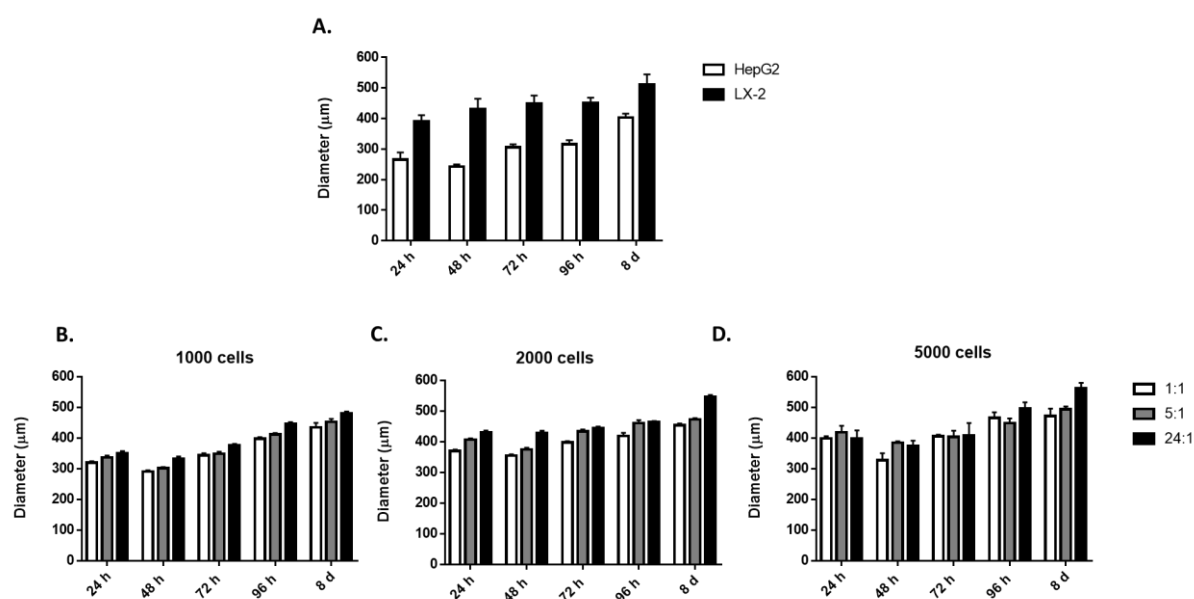


Figure 2.20. Changes in diameter of HepG2/LX-2 multicellular spheroids over time

(A) Diameter of 3D monocellular spheroids of HepG2 or LX-2 cells grown from 1,000 cells per 100 μ l culture medium in CellStar® cell repellent plates for up to 8 d. Data are shown as mean $\pm$ SEM ($n_{\text{replicates}} = 6$). (B-D) Diameter of HepG2/LX-2 multicellular spheroids grown from (B) 1,000, (C) 2,000 and (D) 5,000 cells per 100 μ l culture medium for up to 8 d in CellStar® cell repellent plates. Data are shown as mean $\pm$ SEM ($n_{\text{replicates}} = 6$).

The viability of the multicellular spheroids thus formed was also assessed using CellTiter-Glo® luminescent viability assay. As shown in Figure 2.21, ATP production remained stable throughout the culture period, with no significant differences in viability detected between the differing cell compositions and timepoints tested.

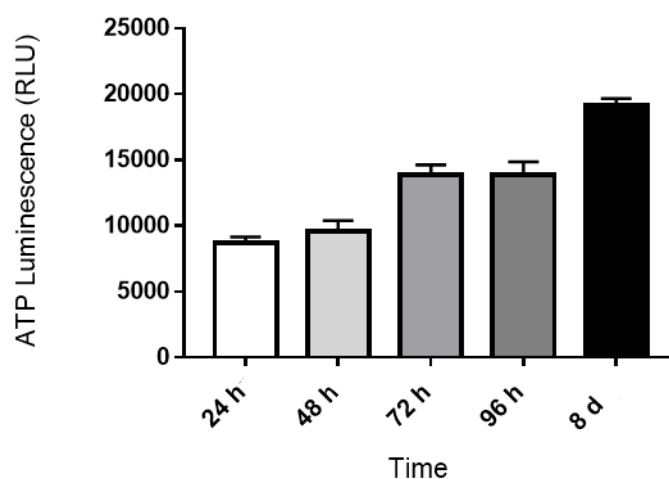


Figure 2.21. Changes in ATP content in HepG2/LX-2 multicellular spheroids over time.

The viability of HepG2/LX-2 multicellular spheroids formed in CellStar® cell repellent plates at a density of 1000 cells in a ratio of 24:1 HepG2/LX-2 were cultured for up to 8 d before viability was determined using the CellTiter-Glo® luminescent viability assay. Data are represented as mean $\pm$ SEM ($n = 6$ replicates).

2.4.4.2 Sensitivity of HepG2/LX-2 multicellular spheroids to detect human hepatotoxins

To determine if the multicellular spheroids were capable of predicting human toxicological responses, the spheroids were exposed to acetaminophen, diclofenac, fialuridine, and trovafloxacin, and viability then assessed. A dose-dependent response curve was plotted (Figure 2.22) and the concentrations of hepatotoxins to cause 50% reduction in cell viability were estimated from the curve (Table 2.3).

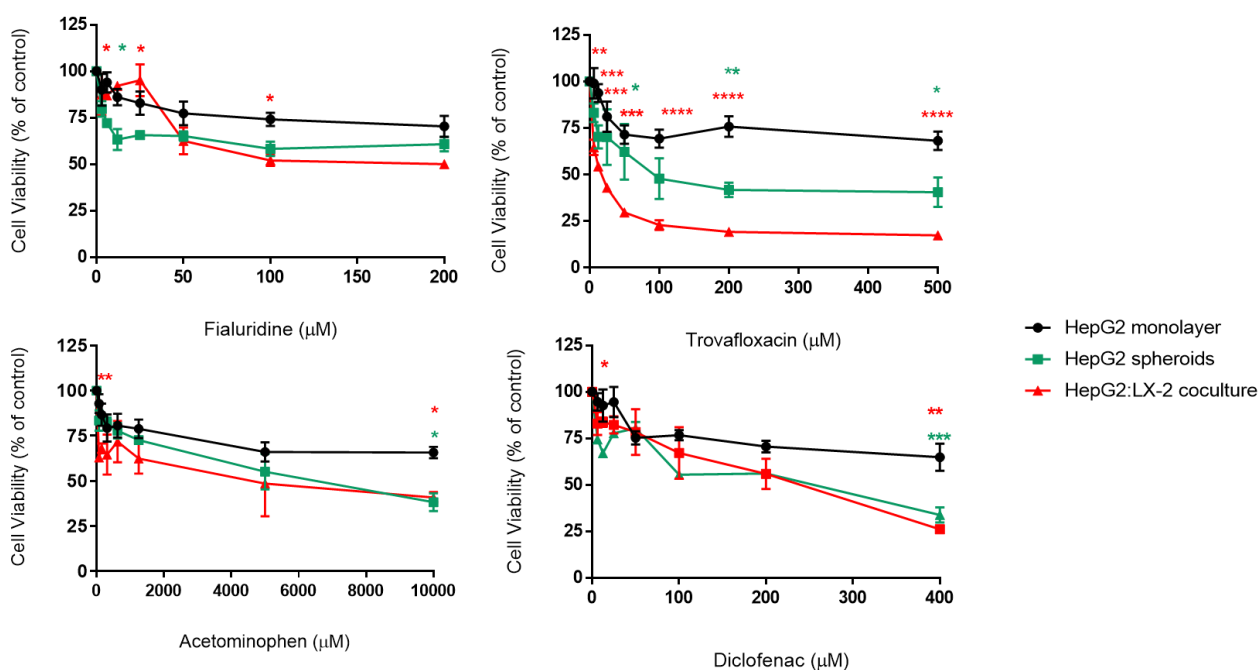


Figure 2.22. Ability of HepG2/LX-2 multicellular spheroids to detect hepatotoxins.

HepG2 2D monolayers and 3D monoculture and multicellular spheroids were cultured for 3 or 10 d and exposed to four hepatotoxins, namely acetaminophen, diclofenac, fialuridine, trovafloxacin and doxorubicin, with repeated dosing at d5 and d12 respectively. Cell viability was determined using ATP quantification and results plotted as a percentage of untreated control. Data represented as mean $\pm$ SEM ($n_{\text{replicates}} = 3$). Multiple t tests with

Bonferroni Dunn method were undertaken. Symbols (*, **, ***, ****) indicate $p < 0.05$, < 0.01 , < 0.001 , and < 0.0001 , respectively.

Table 2.2. Sensitivity of 2D, 3D and multicellular spheroids to a panel of common hepatotoxins

The concentration of hepatotoxin to cause 50% reduction in cell viability was estimated from data plotted in Figure 2.23 and compared to data found in the scientific literature. Abbreviations: PHH, primary human hepatocytes.

Compound	Value at which 50% reduction in viability is observed (μM)			
	HepG2 spheroid - 4- day repeat dosing	HepG2 2D monolayer - 24 h acute dosing	HepG2/LX- 2 multicellular spheroid - 4- day repeat dosing	2D monolayer of PHH - Data from the scientific literature
Fialuridine	>200	>200	>200	>1,000
Trovaflaxacin	>100	>400	>20	~200
Acetaminophen	>8,000	>10,000	>5,000	~20,000
Diclofenac	>250	>400	>200	331

The concentration at which fialuridine would reduce cell viability by 50% was the same across all cultures, i.e. >200 μM . Multicellular spheroids were significantly more sensitive to trovaflaxacin (>20 μM) compared to HepG2 spheroids (>100 μM) and HepG2 monolayers (>400 μM). They were also more sensitive to acetaminophen (>5,000 μM) compared to HepG2 spheroids (>8,000 μM) and HepG2 monolayers (>10,000 μM), however this was non-significant (table 2.4). Multicellular spheroids were significantly more sensitive to diclofenac, i.e. >200 μM v. >250 μM for HepG2 spheroids and >400 μM for HepG2 monolayers.

Table 2.3. Statistical analysis of 2D, 3D and coculture spheroids to detect hepatotoxins

Data represented as mean $\pm$ SEM ($n_{\text{replicates}}=3$). ns denoted non-significant. The symbols * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (multiple t tests with Bonferroni Dunn method).

		1	2	3	4	5	6	7
Fialuridine	2D v. 3D	ns	ns	ns	ns	*	ns	ns
	2D v. Coculture	ns	*	ns	ns	ns	ns	ns
	3D v. Coculture	ns	ns	ns	*	*	ns	ns
Trovafloracin	2D v. 3D	*	**	ns	ns	ns	ns	ns
	2D v. Coculture	****	****	****	****	****	****	**
	3D v. Coculture	ns	ns	ns	*	ns	ns	ns
Acetaminophen	2D v. 3D	*	ns	ns	ns	ns	ns	**
	2D v. Coculture	*	ns	ns	ns	ns	ns	ns
	3D v. Coculture	ns	ns	ns	ns	ns	ns	ns
Diclofenac	2D v. 3D	***	ns	ns	ns	ns	ns	ns
	2D v. Coculture	**	ns	ns	ns	ns	*	ns
	3D v. Coculture	ns	ns	ns	ns	ns	ns	ns

2.4 Discussion

This chapter describes the development and optimisation of a robust and reproducible protocol for forming monoculture and multi-cellular hepatic spheroids from HepG2 and LX-2 cell lines. Whilst the current gold-standard for drug metabolism and toxicity studies is the use of primary human hepatocytes (PHH) [83, 317], 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 in this PhD project. Although when cultured in 2D HepG2 cells exhibit many limitations including loss of liver-specific structure and functionality [83, 318], their ease-of-use as compared to PHH, coupled with a stable phenotype and no donor variation [83, 127], 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 [157].

In this chapter, HepG2 spheroids were cultured and characterised for liver-specific structure and function, using two substrate and scaffold free methods: CellStar[®] cell repellent plates (CRP) and the InSphero GravityPLUS[™] hanging drop system. These plates were chosen as they are easy to acquire, standardised and high throughput in nature, whilst also known to produce reproducible spheroids [319-321]. HepG2 spheroids formed successfully using the two methods (Figure 2.4), with the cells in each well aggregating to form a single spheroid, however spheroids formed from CellStar[®] CRP had distinct advantages over the InSphero[™] system. 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. They were more cost-effective and demonstrated a higher degree of reproducibility in forming 3D HepG2 spheroids. These factors, coupled with their amenability to both high-throughput screening, cost-effectiveness and repeated dose regimens, made me conclude that 3D spheroids formed in CellStar[®] CRP plates were the most promising models for large-scale toxicological screening of NBMs [84]. To the best of my knowledge, HepG2 spheroids formed in CellStar[®] CRP plates have not been previously reported in the scientific literature [228], making my work a step forward in the field. The lowest cell seeding density tested (1000 cells per 100 μ l culture medium), produced the most uniform, circular spheroids with the most consistent morphology throughout culture period, with diameters ranging from $294.26 \pm 10.43 \mu\text{m}$ at d3, to 647.95

$\pm 16.86 \mu\text{m}$ after 32 d (Figure 2.5 and 2.9). These results are consistent with other studies where PHH and other immortalised liver cell lines were grown in round bottom non-adherent plates [299, 307].

One significant issue with using spheroid for the toxicity screening of drugs and NBMs is the fact that necrosis often occurs within the spheroid core, particularly for larger spheroids, something that can interfere with toxicity readouts. Whilst few studies exist to fully underpin the extent of necrosis in hepatic spheroids, is estimated that the distance essential materials like oxygen and nutrients can diffuse through them is approximately 100 – 150 μm [322-324], however, this is highly dependent on density and culture duration, and the size and time at which necrotic core development occurs has not been specifically determined. Other factors like cell type, culture type and potential scaffold interactions are all important issues which further complicate necrotic core development in spheroid models. One possible means of determining spheroid health is by assessing ATP production. Following analysis using the CellTiter-Glo[®] ATP quantification viability assay, it was determined that the optimised HepG2 spheroids remained viable, producing ATP for the 31d culture period, with a drop-off in ATP production observed only after 19 d (Figure 2.8). The live/dead immunofluorescent assay confirmed such results (Figure 2.15). However, it must be noted within the limitations of this study that the live/dead fluorescence assay is not an optimal method of determining viability of larger spheroids, as it is significantly limited by the ability of the dyes to penetrate larger spheroids with more dense, compact structures. This is an issue often cited in literature, with live/dead most used for spheroids of smaller starting densities, cultured for shorter periods, and most commonly for high throughput screening applications [171, 306, 325].

To overcome this limitation, histological analysis was also carried out. H&E staining showed that HepG2 spheroids grown from a cell seeding concentration of 1,000 cells per 100 μl culture medium did not possess a necrotic core until 27 d, in contrast to spheroids grown from larger seeding concentrations, where cell death and loss of spherical morphology was detectable as early as 11 d, with noticeable patches of cell death and necrotic cores appearing at 15 d (Figure 2.9). From this I concluded that spheroids which exceed this critical size of approximately 600 μm were likely to be structurally unstable with pockets of necrosis and should be deemed unsuitable for further experimentation. Thus, for the rest of the project, 3D spheroids grown from a cell seeding concentration of 1,000 cells per 100 μl culture medium were used. Histological analysis also revealed a cuboidal cellular morphology, with direct cell-cell contacts visible, recapitulating the structure of the *in vivo* liver [326] (See

Supplementary Figure 2.4). These factors overcome many of the key phenotypic and structural model limitations observed when hepatocytes are cultured in 2D [327-329].

Another key feature of *in vivo* hepatocytes is their structural and functional polarisation [84, 89], as observed in Supplementary Figure 2.5. As demonstrated by immunofluorescent staining and confocal imaging, formation of secondary bile canaliculi-like structures was observed in the HepG2 spheroids developed, starting from day 4, with these structures further elongating and joining together by day 18, mimicking work conducted by Ramaiahgari et al [157], Sharma et al [330] and Fleddermann et al [283] (Figure 2.10 and 2.11). These structures confirm spheroid structural polarity. Polarisation features observed in 3D spheroid could not be found in 2D monolayers formed by HepG2 cells. The polarisation features of the HepG2 spheroids formed were also identified to be functional, with the MRP2 transporter (a transporter localised to the canalicular membrane of *in vivo* hepatocytes) expressed on the canalicular membrane. Expression of the MRP2 transporter is vitally important as not only does it play crucial roles in liver homeostasis, but also in the movement of drugs and materials in and out of hepatocytes, with dysfunction of MRP2 and other liver transporters ultimately leading to hepatotoxicity and/or liver damage [331-333]. Thus, the data obtained from the structural characterisation seemed to suggest that the HepG2 spheroids formed did in fact recapitulate features of the *in vivo* liver. Because of this, it was then decided to undertake functional characterisation in order to see if HepG2 spheroids were in fact capable of being functional, with comparisons made to corresponding 2D HepG2 cultures.

The production and secretion of various materials including albumin, ammonia and urea in hepatocytes is a key characteristic of their functionality and highly indicative of long-term performance [334]. From undertaking a human serum albumin ELISA, it could be confirmed that HepG2 spheroids were in fact able to synthesise and secrete albumin, with significantly higher quantities of albumin secreted in the spheroids compared to 2D cultures (Figure 2.12). The synthesis of albumin in HepG2 spheroids indicates synthetic and metabolic function as production of albumin is vitally important for hepatocyte physiological function *in vivo* [85]. The ability of hepatocytes to produce and transport bile is another essential role for properly predicting hepatotoxicity. Functionality of the MRP2 transporter expressed within the 3D spheroids formed was confirmed by the accumulation of the fluorescent bile substrate in the canalicular membranes, with limited substrate observed outside the cell, thus indicating active transport of substrate out of cell through canaliculi (Figure 2.14). Further confirmation was observed following the blocking of this transporter with an MRP2 inhibitor, MK571. These results on MRP2 transport functionality in 3D spheroids are in agreement with, those

reported by Gaskell *et al* (C3A cells) [307], Ramaiahgari *et al* (HepG2 cells) [335], and Mueller *et al.* (HepG2 cells) [336] illustrating that this phenomenon occurs in multiple different hepatic cells lines when they are cultured in 3D. All these results confirm and build on the evidence that HepG2 cells cultured as spheroids have superior functionality to HepG2 2D cultures. Experiments with the autofluorescent compound doxorubicin, also demonstrated that it would be possible for hepatotoxic compounds to successfully penetrate the dense layers of the spheroids. Z-stack images from confocal microscopy indicate that doxorubicin did in fact penetrate the tightly packed cell layers in the spheroids, with doxorubicin fluorescing throughout spheroid to the middle core section, as well as being visible on the periphery (Figure 2.16). This result was in agreement with work undertaken by Song *et al.* [337-340] and indicated that spheroid responses to compound exposure would be determined by the response of all the cells in the spheroid and not just a small subsection. A major use 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. Both drug compounds and NBMs induce drug induced liver injury by a variety of different mechanisms. It has been suggested that many compounds induce liver injury through damaging the mitochondria, including fialuridine, diclofenac, troglitazone and amiodarone [340-344]. Other hepatotoxins can cause transporter dysfunction or loss of hepatocyte structural integrity, such as tolcapone, nefazodone, troglitazone and diclofenac [341-343]. Because of this, the secretion of bile is hindered and does not effectively get secreted into bile ducts. This causes toxic substances to build-up around hepatocytes [340, 343, 345-347]. As illustrated in this study, HepG2 spheroids demonstrated sufficient localisation of canalicular-like structures, which were functional, meaning that the spheroids could successfully predict any cholestatic compound which could potentially interfere with bile transport. Outside of mitochondria and bile duct dysfunction, it is often metabolites of certain compounds which can cause damage to the liver, either by being directly hepatotoxic or from the formation of adducts with proteins in the liver. Common compounds which induce toxicity in this manner include tolcapone, troglitazone, nefazodone, acetaminophen and diclofenac [118, 348]. Other studies have suggested that immune response can be involved, heightening the toxicity of a variety of hepatotoxins, including diclofenac and trovafloxacin [157, 349, 350]. However, for any of the aforementioned damages to be detected appropriately, sufficient cell communication and signalling must be present in the model, along with the ability to initiate immune responses. A final consideration to consider is longevity of model. As various hepatotoxins take time to induce toxicity or require multiple doses, a 4-d repeated dosing strategy was used in this study. This regime was used

to better mimic the dosing experienced by patients. For 2D monocultures, a standard 24 h treatment was used to avoid cells becoming over-confluent and dying. 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 Table 2.4.

Table 2.4. The hepatotoxins used in this study, the liver injury they cause and suggested mechanism.

Compound	Drug-induced liver injury undetected in:	Clinical drug-induced liver injury	Suspected mechanism of action
Diclofenac	Clinical Trials	Concerning DILI, acute or chronic liver disease	Reactive metabolites, immune allergies, mitochondrial dysfunction, transporter inhibition
Fialuridine	Pre-Clinical Screening	Fatal liver injury	Mitochondrial dysfunction
Acetaminophen	Clinical Trials	Fatal liver injury	Reactive metabolites
Trovafloxacin	Clinical Trials	Hepatotoxicity and liver failure	Inflammatory stress

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 (Figure 2.18). Spheroid culture time was also found to have no effect on this result with spheroids cultured for both 5 and 12 d exhibiting no significant differences in sensitivity (Figure 2.18). 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 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 [84, 351, 352]. For example, Li *et al.* used 3D hepatic spheroids comprised of PHH to assess the hepatotoxic potential of 100 known hepatotoxic drugs, and found that in comparison to conventional 2D liver monolayers, the 3D spheroids were significantly more sensitive to detecting hepatotoxicity [353]. Bell *et al.* have detailed similar findings [302], as have Ramaiahgari *et al.*, who used HepG2 spheroids [354, 355]. Whilst HepG2 spheroids were more predictive than HepG2 2D monolayers to all hepatotoxins, from values based in literature and from my own experiments, estimated values at which 50% cell viability is reduced were only comparable to PHH for trovafloxacin and diclofenac treatments, and not for acetaminophen or fialuridine. It can be hypothesised that the mechanisms for which both acetaminophen and fialuridine exhibit their hepatotoxicity plays a role in this variation. HepG2 spheroids were shown to exhibit toxicity in HepG2 spheroids at a much lower concentration than what is detailed in the literature for PHH. With regard to acetaminophen, it is suggested that metabolites of this compound cause liver damage, by either being directly hepatotoxic or by forming adducts with various liver proteins. Other hepatotoxins induce toxicity via the same mechanism include tolcapone, perhexiline, nefazodone and amiodarone [302, 355-357]. As CYP enzyme activity and the ability to metabolise xenobiotic compounds were not probed in this thesis, it can be hypothesised that the variation observed between HepG2 spheroids and PHH may potentially stem from the lack of drug metabolites forming in HepG2 spheroids and the ability of the spheroids to observe their toxic effects [358]. Furthermore, it has also been suggested that various other compounds induce hepatotoxicity through mitochondrial damage, including amiodarone, troglitazone, and the hepatotoxin used in this study, fialuridine [337, 339, 340]. In this study variation observed between HepG2 spheroids and PHH when treated with fialuridine. The presence of functional mitochondria in HepG2 spheroids was not probed in this study, and the inclusion of mitochondria assessment in further work would be of great benefit to underpinning the toxicity of fialuridine in the spheroid model presented. Other studies have suggested that immune response is involved in hepatotoxicity, going so far as to accentuate hepatotoxicity of certain compounds. Both diclofenac and trovafloxacin, the two hepatotoxins used in this study which exhibited similar sensitivity to PHH, are both compounds which display evidence of inflammatory or immune-mediated toxicity [344, 348]. For this hepatotoxicity to be successfully detected in spheroids, proper cell communications and signalling must be present in the model, as well as capabilities to either alert or initiate immune responses. The comparable sensitivity of the HepG2 spheroids and PHH suggests that this is indeed the case in the HepG2 spheroids, and that cell communication and signalling may be comparable to *in vivo*.

After successful culture and characterization of single cell hepatic spheroids, hepatic stellate cells (LX-2) were incorporated into the model in order to determine if adding a second, non-parenchymal cell (NPC) line into the model would increase its sensitivity. For decades, most hepatic *in vitro* models were comprised of hepatocytes or their immortalized counterparts alone [84], however, NPCs such as hepatic stellate cells, Kupffer cells and LSECs are becoming more widely used due to the critical physiological roles they play in the liver, specifically during liver damage and regeneration [84, 351, 352], and in recent years these cell types are being incorporated more and more into hepatic spheroid models. To date, there is growing evidence to suggest that inclusion of NPCs into *in vitro* liver models not only assists in the maintenance of liver-specific phenotype and function in hepatocytes, but also in how the models responds to hepatotoxins [162, 163, 359, 360]. Therefore, in order to recapitulate the liver as accurately as possible *in vitro*, it may be vital to include an NPC line to increase the sensitivity of the model to hepatotoxins. It was hypothesised that LX-2 cells, an immortal human hepatic stellate cell line and the second most abundant cell type of the liver lobule, would be a useful cell line to add to the model, in a similar manner to work undertaken by Pingitore *et al.* [302]. HSCs are a major fibrogenic type of cell in the liver who contribute to the accumulation of collagen during chronic liver disease, with recent studies also suggesting that HSCs may also be mesenchymal in origin [354, 355]. During normal liver function, HSCs maintain a non-proliferative and quiescent phenotype, however following damage they transdifferentiate, producing various ECM proteins [302, 355-357]. To determine the ideal hepatocyte to HSC ratio, multicellular hepatic spheroids were cultured from three ratios, 1:1, 5:1 and 24:1 (hepatocyte: stellate), and three densities, 1,000, 2,000 and 5,000 cells per 100 μ l culture medium, as well as LX-2 and HepG2 spheroids alone (Figure 2.19). Progressive adhesion of cells began after 24 h, with intact spheroids successfully forming with all starting densities and ratios, with LX-2 spheroids appearing less compact than the HepG2 spheroids. The compactness of the multicellular spheroids was most notable in the most physiologically relevant ratio, 24:1 (HepG2/LX-2). No decrease in viability was observed in the co-culture spheroids over an 8-day time course, with ATP levels remaining constant (Figure 2.21). The next experiments were to underpin if incorporating an NPC cell-line into the spheroid model would increase its sensitivity to the same panel of hepatotoxins previously tested. Due to the nature of LX-2 cells, it could be hypothesised that incorporating these cells into a multicellular spheroid model with HepG2s could create a more *in vivo*-like microenvironment, in turn increasing the model sensitivity to a panel of common hepatotoxins. Enhanced ability of the multicellular spheroids to detect hepatotoxins compared to the HepG2 spheroid model varied across the different compounds

(Figure 2.22). The same sensitivity was observed for fialuridine in both models, with the approximate value at which 50% reduction in cell viability occurring was $>200\ \mu\text{M}$. Multicellular spheroids were significantly more sensitive to trovafloxacin ($>20\ \mu\text{M}$) compared to HepG2 spheroids ($>100\ \mu\text{M}$), as they were for acetaminophen, i.e. $>5000\ \mu\text{M}$ compared to HepG2 spheroids, $>8000\ \mu\text{M}$. Multicellular spheroids were significantly more sensitive to diclofenac, i.e. $>200\ \mu\text{M}$ and $>250\ \mu\text{M}$ for HepG2 spheroids. Whilst the incorporation of HSCs into a multicellular spheroid model with HepG2 did increase the sensitivity of spheroids to hepatotoxins, only diclofenac exhibited similar results to PHH. It is possible that including the NPC LX-2 cell line into the model did in fact create a more *in vivo*-like environment which allowed the hepatotoxins to exert a greater hepatotoxic effect, however further functional characterisation such as assessment of mitochondrial activity and CYP enzyme activity would need to be undertaken on the multicellular model to further probe this hypothesis.

2.6 Conclusion

- HepG2 cells can be cultured in non-adherent plates, producing monoculture or multi-cellular spheroids which remain viable for prolonged periods of time, while maintaining their spherical morphology. The best methodology is based on CellStar™ cell repellent plates.
- HepG2 spheroids display greater *in vivo* relevance than their 2D monolayer counterparts, including an *in vivo*-like structure and function, cell polarisation, increased albumin secretion and direct cell-cell contacts.
- HepG2 spheroids can predict the human hepatotoxicity of a variety of hepatotoxins, with increased sensitivity as compared to conventional 2D monolayers observed for three out of the four hepatotoxins tested, making them a more physiologically relevant model for NBM toxicity screening.
- Creating a multicellular hepatic spheroid increases the susceptibility of the model to hepatotoxins, with the multicellular model exhibiting greater sensitivity to three out of the four hepatotoxins tested, compared to monoculture spheroids

Chapter 3

Biological relevance of 3D hepatic
spheroid models for NBM assessment

Chapter 3 - NBMs physico-chemical characterization and exposure to Hepatic Spheroid Model and 3D Rendering of NBMs accumulation

Chapter Aim:

To determine the biological relevance of the 3D hepatic spheroids developed in Chapter 2. Here, the term “biological relevance” indicates the ability of 3D spheroid models to provide qualitative information on NBM accumulation in the human liver that is comparable to that predicted by *in vivo* studies in animals.

3.1 Introduction

In recent decades there has been great interest in using NBMs in medical technology. As compared to conventional treatments, NBMs offer several advantages, including selective accumulation to targeted tissues (e.g. tumour sites) and subsequent increased delivery of drugs/diagnostic materials, enhanced efficacy and reduced toxicity. Despite this, one major challenge for NBMs is their heterogeneous penetration in solid tumours [361], an issue that has been observed previously with both Abraxane[®] and Doxil[®], the two primary FDA-approved NBMs [362, 363]. Today, most of the justification for using NBMs is centred around the EPR effect (enhanced permeability and retention effect), according to which NBMs preferentially accumulate in the tumour tissues and inflammation sites due to their leaky vasculature and abnormal lymphatic system function. However, abnormalities in vascular and lymphatic function are heterogenous across the tumour tissue/inflammation site, causing heterogeneous accumulation of NBMs and impacting on their ability to deliver an effective treatment. When discussing the ability of NBMs to reach their targets, their ability to penetrate the ECM and be uptaken by the targeted cells must also be mentioned [290, 364, 365].

Having assessed how the spheroids developed in Chapter 2 work on their own, in this chapter I describe their ability to work with a panel of representative NBMs with applications in medical technology, and their ability to provide qualitative information on the accumulation in the liver and interaction with hepatocytes, and also determined whether this information was predictive of *in vivo* studies of similar NBMs, or where available, the same NBMs. The current processes for assessing NBM tissue penetration and cellular internalization, relies on

reductionist *in vitro* 2D models and animal models. The latter are not only expensive and time-consuming models, but also poorly representative of the human environment. Issues also arise when using animal models for accurately studying the NBM penetration at cellular/tissue level; thus, these models are less and less employed for studying NBM tissue penetration and cellular uptake. 3D spheroids have emerged as an additional and valuable tool for assessing liver-specific toxicity of NBMs, due to their ability to better mimic the key properties of human tissues; however to date, their use in predicting NBM liver accumulation and cellular uptake is still in its infancy.

Spheroids were exposed to three NBMs; (1) a metallic NBM, namely a 20nm AuNP, and (2) two organic NBMs, (a) an IR-780 dye-loaded liposome, LipImage™815 (referred to as LipImage in this study), and (b) an NR668 dye-loaded PEBCA polymeric nanoparticle (referred to as PACA), with each of these materials representative of a NBM class currently in trials or used in the clinic. AuNPs (representing the metallic NBM class) are generally regarded as safe; however, many papers have suggested that AuNPs may still cause off-target side effects, stimulating inflammatory responses and accelerate stress-induced apoptosis [366-368]. LipImage represents liposomes. The latter are formulated to limit off-target side effects that are observed with the free drugs they encapsulate; nevertheless, due to their pharmacokinetics, liposomes can end up sequestered in organs of the mononuclear phagocyte system, including the liver. Liposomes have been shown to impair liver function by attenuating phagocytic capacity and causing depletion of macrophages [369]. Finally, PACA represents polymeric NBMs. These have also been shown to non-specifically accumulate in the liver [370]. As several studies have demonstrated that NBM physico-chemical properties, i.e., their size, charge, surface coating, shape and overall composition, play critical roles in their internalization and biodistribution in tissues, physico-chemical characterisation of the NBMs tested in this Chapter was first performed.

NBM internalization and interaction with liver cells was assessed using confocal microscopy and 3D rendering image analysis, and the results obtained compared to results obtained in conventional 2D cultures of HepG2 and, where possible, to *in vivo* data available from the scientific literature.

3.2 Materials and Methods

Unless stated otherwise, all materials relating to this chapter were purchased from Sigma Aldrich, Ireland.

3.2.1 NBMs and their physico-chemical characterization (PCC)

The NBMs used in this study were (Figure 3.1):

- A. 20nm PEGylated AuNP (purchased from nanoComposix, San Diego, California);
- B. LipImage™815, a liposome encapsulating IR780 dye (kindly supplied by CEA-LETI, France);
- C. PEBCA, a polymeric nanoparticle of the PACA family, loaded with NR668 dye (kindly supplied by SINTEF, Trondheim, Norway).

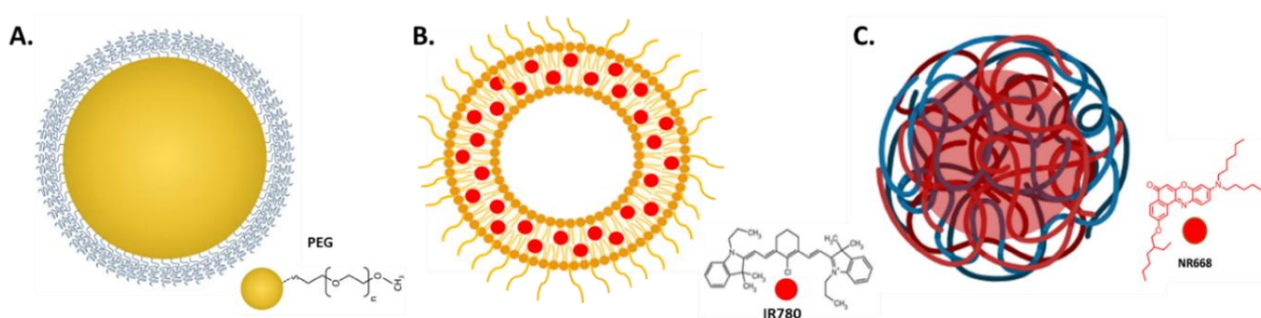


Figure 3.1 Schematic of the NBMs used in this study.

(A) 20nm PEGylated AuNP, (B) LipImage™815 (LipImage), and (C) NR-668 PEBCA (PACA). Illustrations not in scale.

The AuNP (Gold Nanospheres, PEG, BioPure™, Product Number: AUGB20) were purchased commercially from nanoComposix (San Diego, California) and were provided at a mass concentration of 1.00 mg/ml and a molar particle concentration of 2.5×10^8 particles (mol/l). Particles were 20nm 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. In house characterization data for AuNP compared to supplied data is detailed in Results Section 3.3.1 below (Table 3.2). Characterization data kindly provided by nanoComposix can be view in Supplementary Figure S3.1.

LipImage, the IR780 dye-loaded liposome, was kindly provided by CEA-LETI (France) at a particle concentration of 95 mg/ml (9.5%) and a dye concentration (HPLC) of 239.5 μ M (252 μ M/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 < 1UI/ml (within acceptable amount). In house characterization data for LipImage compared to supplied data is detailed below (Table 3.3). Characterization data kindly provided by CEA-Leti can be view in Supplementary Figure S3.2.

NR 668-loaded PEBCA NBM (PACA) (Particle REF-24-3), was kindly provided by collaborators at SINTEF (Trondheim, Norway) at a stock particle concentration of 105 mg NP/ml. PACA has a particle size of 52 nm. Dispersion media used was 1mM HCl in sterile distilled water. PACA was also deemed sterile and endotoxin free. In house characterization data for this NBM compared to supplied data is detailed below (Table 3.4). Characterization data kindly provided by CEA-Leti can be view in Supplementary Figure S3.3.

For NBM characterization, all samples were stored at 4°C and equilibrated to RT before characterization, which was kindly undertaken by Gabriele Vella, Nanomedicine and Molecular Imaging Group, 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 (EUNCL-PCC-023 <http://www.euncl.eu/about-us/assay-cascade/>) was used [371]. 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’ (<http://www.euncl.eu/about-us/assay-cascade/>)

[cascade/PDFs/Prescreening/EUNCL-PCC-001.pdf?m=1468937875&\)](#) [372], 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.

3.2.2 Cell culture

HepG2 and LX-2 cells were maintained as described in Chapter 2, section 2.2.1. 2D and 3D cultures were formed as described in Chapter 2, sections 2.2.2 and 2.2.3 respectively. HepG2 monolayers and spheroids are treated with the aforementioned NBMs (characteristics listed above, Section 3.3.1). As all materials were deemed sterile and endotoxin free, they could be used in cell culture.

3.2.3 Exposure to NBMs

2D monolayers were left to adhere for 24 h before culture medium was removed, cells washed with pre-warmed PBS and NBM treatments added for 24 h. HepG2 spheroids were cultured for 8 d (with culture medium changed every 3 d) before culture medium was removed, spheroids were washed with pre-warmed PBS and exposed to NBMs for 24 h. Several concentrations of the chosen panel of representative NBMs were tested; these are reported in Table 3.1. Characterisation information for these NBMs is listed above in Section 3.3.1. All NBMs were dispersed in low glucose DMEM, supplemented with 10% Foetal Bovine Serum (FBS) and 1% penicillin streptomycin. Untreated and no primary antibody controls were included in experimental design.

Table 3.1. Nanoparticles chosen for this study and concentrations used.

NBMs	Concentration (µg/ml)
AuNP	10, 20 and 30
LipImage™815	100, 200 and 500
NR668 PEBCA (PACA)	10, 20 and 30

3.2.4 Immunofluorescent staining

Immunostaining and confocal microscopy were undertaken on 2D cultures and 3D spheroids, exposed to varying concentrations of the chosen NBMs. For 2D monolayers and 3D spheroids, culture medium was removed, and cell layer washed with pre-warmed PBS. After fixation in 3.7% PFA for 10 min at room temperature (RT), specimens were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked in 1% BSA in PBS for 1 h. Specimens were then stained with anti-PEG primary antibody (ab170969) (ABCAM, Ireland) (1:500 in 0.1% Triton X-100 in PBS), incubated overnight (O/N) at 4°C in the dark, before washing and incubation with appropriate secondary antibodies. For LipImage and AuNP, Alexa Fluor 488 goat anti-mouse secondary antibody (1:500) was used, with nuclei and actin stained using Hoechst 33342 (1:1000) and rhodamine phalloidin (1:50) respectively (both Invitrogen, Fisher Scientific, Ireland). Staining undertaken at RT in the dark for 1 h. As PACA was loaded with NR668 (Nile red dye, a fluorescent molecule emitting in the red region of the visible spectrum), rhodamine phalloidin was not used to avoid overlap of signals, and only cell nuclei were stained with Hoechst 33342 (1:1000) for 1 h at RT in the dark. 2D monolayers were stained with Hoechst 33342 (1:1000) and rhodamine phalloidin (1:50). Staining undertaken at RT in the dark for 1 h. Following staining, specimens were washed in pre-warmed PBS and mounted on Idibi μ -Slide angiogenesis slides (Ibidi GMBH, Germany) using SlowFade™ Gold Antifade Mountant (Thermo Fisher, Ireland). Qualitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, Trinity College Dublin, using a Leica SP8 confocal microscope with a 20x dry objective. Image processing undertaken using LAS X software. Z-stack 100 steps, physical length 124.9 μ m Total exposures: (300 channels, 100 frames).

3.2.5 3D rendering of confocal images

3D reconstruction of z-stack images acquired using LSCM (Section 3.3.5) was carried out by uploading images to Icy v software (Version 2.0.3.0) (open community platform for bioimage informatics, <http://icy.bioimageanalysis.org/>) and generating 3D volumes [373]. Reconstruction parameters were consistent across all NBMs. Z-stacks of 100 steps, physical length 124.9 μ m. Total exposures: (300 channels, 100 frames) were imputed into ICY, with 35 images used for rendering.

3.3 Results

3.3.1 Characterization of chosen NBMs

The quantitative analysis of the physicochemical characteristics of any NBM is an essential step in order to select a suitable formulation to bring forward in any study. In order to determine if NBMs had remained stable during transit, NBM characterization was undertaken for the AuNP, LipImage and PACA NBMs prior to any further experimentation, with results compared to characterization data from supplier. NTA was undertaken to assess NBM hydrodynamic diameter, using the NS500 Nanosight (Software Version 3.2) (Malvern-Panalytical, UK), according to protocols previously published by our group [371, 374] and which are now well established as validated protocols for NBM under the EUNCL [375, 376]. Results from NTA were then confirmed with measurements obtained using dynamic light scattering (DLS) (Malvern Nano- ZS, Malvern-Panalytical, UK), following the EUNCL protocol for DLS size analysis [372]. Values obtained for both NTA and DLS were compared to datasheets provided by suppliers to assess if NBMs remained stable during transport.

The AuNP (20nm Gold Nanospheres, PEG, BioPure™, Product Number: AUGB20) were purchased commercially from nanoComposix (San Diego, California) and were provided at a mass concentration of 1.00 mg/ml and a molar particle concentration of 2.5×10^8 particles (mol/l) in MilliQ H₂O. In house characterization data for AuNP compared to supplied data is detailed below (Table 3.2). NTA reported a mean size of 43.4 nm, which closely resembles the DLS measurements of 42.45 nm. DLS measured an average zeta potential -24 mV at pH 7, making the AuNP anionic. As observed in Figure 3.2, some small aggregates are present at approximately 100 nm. PDI index, obtained from DLS, is 0.102, making AuNP monodispersed. In house characterization is closely related to supplier values provided (Supplementary Figure S3.1).

Table 3.2. Summary and comparison of characterization data by NTA and DLS for AuNP

AuNP Measured Parameter	Supplier Value	TCD Value
Mean hydrodynamic size (DLS)	45.5 nm	42.45 nm
Mean hydrodynamic size (NTA)	Not provided	43.4 nm
Polydispersity Index (PDI) (DLS)	Not provided	0.102
Zeta Potential	-26 mV	-26.4 mV

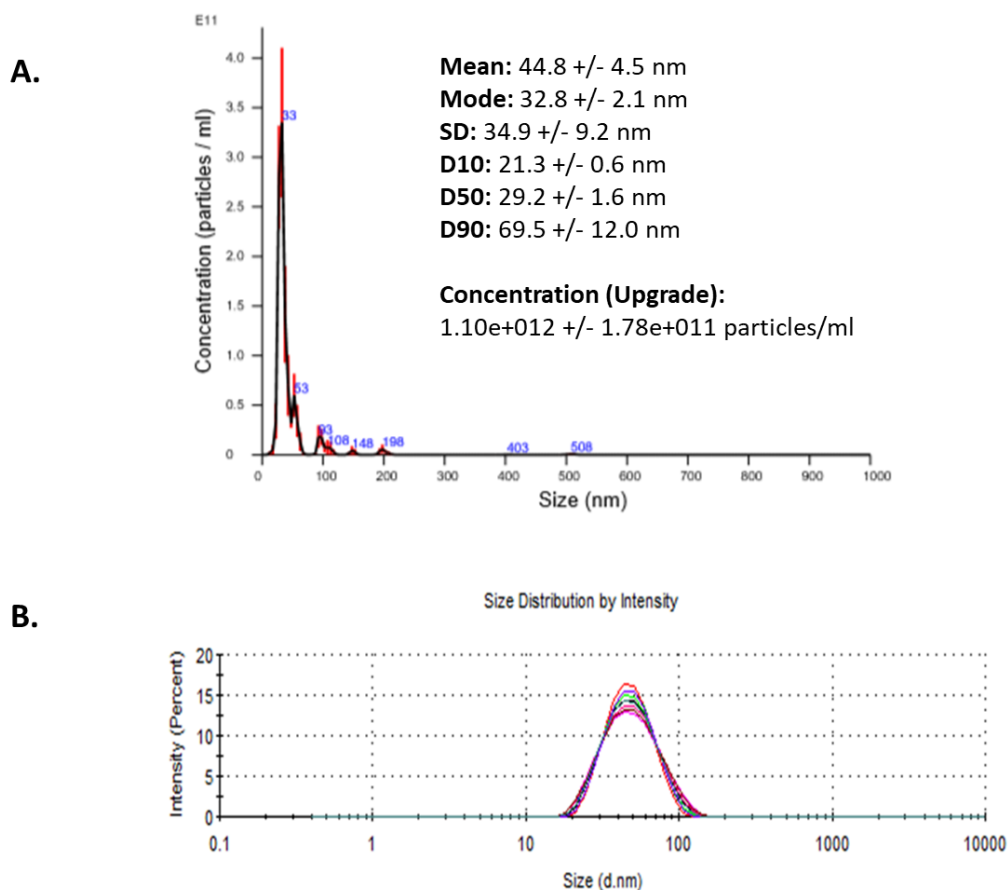


Figure 3.2. NTA and DLS analysis of AuNP.

(A) Nanoparticle tracking analysis (NTA) size versus concentration graph. AuNP was diluted to 10 µg/ml in particle-free water (Sigma Aldrich, Ireland) and analysed through 5, 60 s recordings. (B) Dynamic light scattering (DLS) graphs depicting size distribution and zeta potential at pH 7.

The second NBM was LipImage, the IR780 dye-loaded liposome was provided by CEA-LETI (France), at a particle concentration of 95 mg/ml (9.5%) and a dye concentration (HPLC) of 239.5 μ M (252 μ M/100 mg particle). Dispersion media used was 154 mM NaCl and ascorbic acid (1.75 g/l). In house characterization data for LipImage compared to supplied data is detailed below (Table 3.2). NTA reported a mean size of 50.72 nm, with DLS measurements reporting a mean size of 72.7 nm. As observed in Figure 3.3, a small aggregate peak is observed at 163 nm. PDI index, obtained from DLS, is 0.11, making AuNP monodispersed. In house characterization is closely related to supplier values provided (Supplementary Figure S3.2).

Table 3.1. Summary and comparison of characterization data by NTA and DLS for LipImage.

LipImage	Supplier Value	TCD Value
Mean hydrodynamic size (DLS)	52.2 nm	50.72 nm
Mean hydrodynamic size (NTA)	Not provided	72.7 nm
Polydispersity Index (PDI) (DLS)	<0.102	0.11

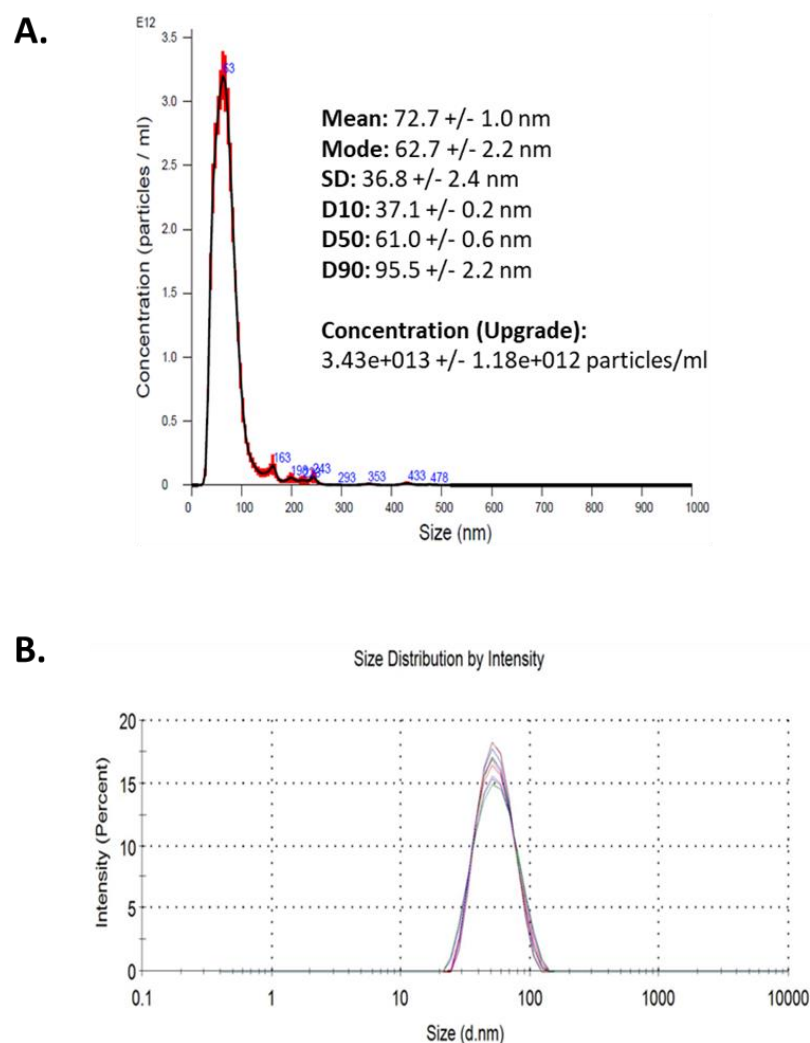


Figure 3.3. NTA and DLS analysis of LipImage.

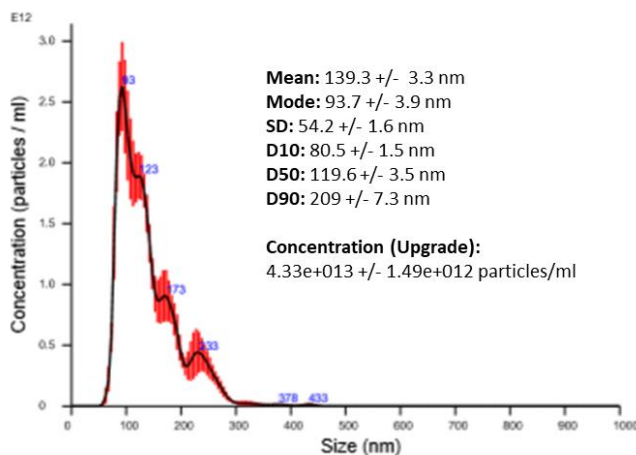
(A) Nanoparticle tracking analysis (NTA) size versus concentration graph. LipImage was diluted to 10 $\mu\text{g/ml}$ in particle-free water (Sigma Aldrich, Ireland) and analysed through five 60 s recordings. (B) Dynamic light scattering (DLS) graphs depicting size distribution.

The third and final NBM was the NR 668-loaded PEBCA NBM (PACA) (Particle REF-24-3), provided by collaborators at SINTEF (Trondheim, Norway) at a stock particle concentration of 105 mg NP/ml. Dispersion media used was 1mM HCl in sterile distilled water. In house characterization data for this NBM compared to supplied data is detailed below (Table 3.4). NTA reported a mean size of 72.7 nm, and DLS measurement of 164.7 nm. DLS measured an average zeta potential -3.6 mV at pH 7, making PACA neutral. PDI index, obtained from DLS, is 0.108, making AuNP monodispersed. In house characterization is closely related to supplier values provided (Supplementary Figure S3.3).

Table 3.2. Summary and comparison of characterization data by NTA and DLS for NR668 PEBCA.

PACA Measured Parameter	Supplier Value	TCD Value
Mean hydrodynamic size (DLS)	178	164.7
Mean hydrodynamic size (NTA)	Not provided	72.7
Polydispersity Index (PDI) (DLS)	0.28	0.18
Zeta Potential	-3.6 mV	Not provided

A.



B.

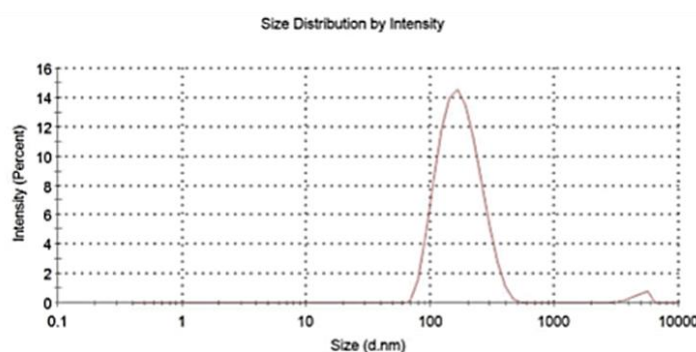


Figure 3.4. NTA and DLS analysis of NR668 PEBCA (PACA).

(A) Nanoparticle tracking analysis size versus concentration graph. PACA was diluted to 10 $\mu\text{g/ml}$ in particle-free water (Sigma Aldrich, Ireland) and analysed through five 60 s recordings. (B) Dynamic light scattering graphs depicting size distribution and zeta potential at pH 7.

3.3.2 Interaction of NBMs in 2D monolayers of HepG2 cells

Before assessing the 2D monolayer, a pilot set of control experiments was undertaken to assess the hepatocytes response and morphological response in 2D and subsequently if they could be successfully imaged using an anti-PEG antibody. Untreated and no primary antibody controls were included in experimental design as a means of validating antibody which would be used throughout the rest of this chapter. No positive staining or background was observed in either control (Figure 3.5), therefore antibody was appropriate to use throughout the monolayer and spheroid experiments. Monolayer and spheroid incubation, observation and staining was carried out following controls.

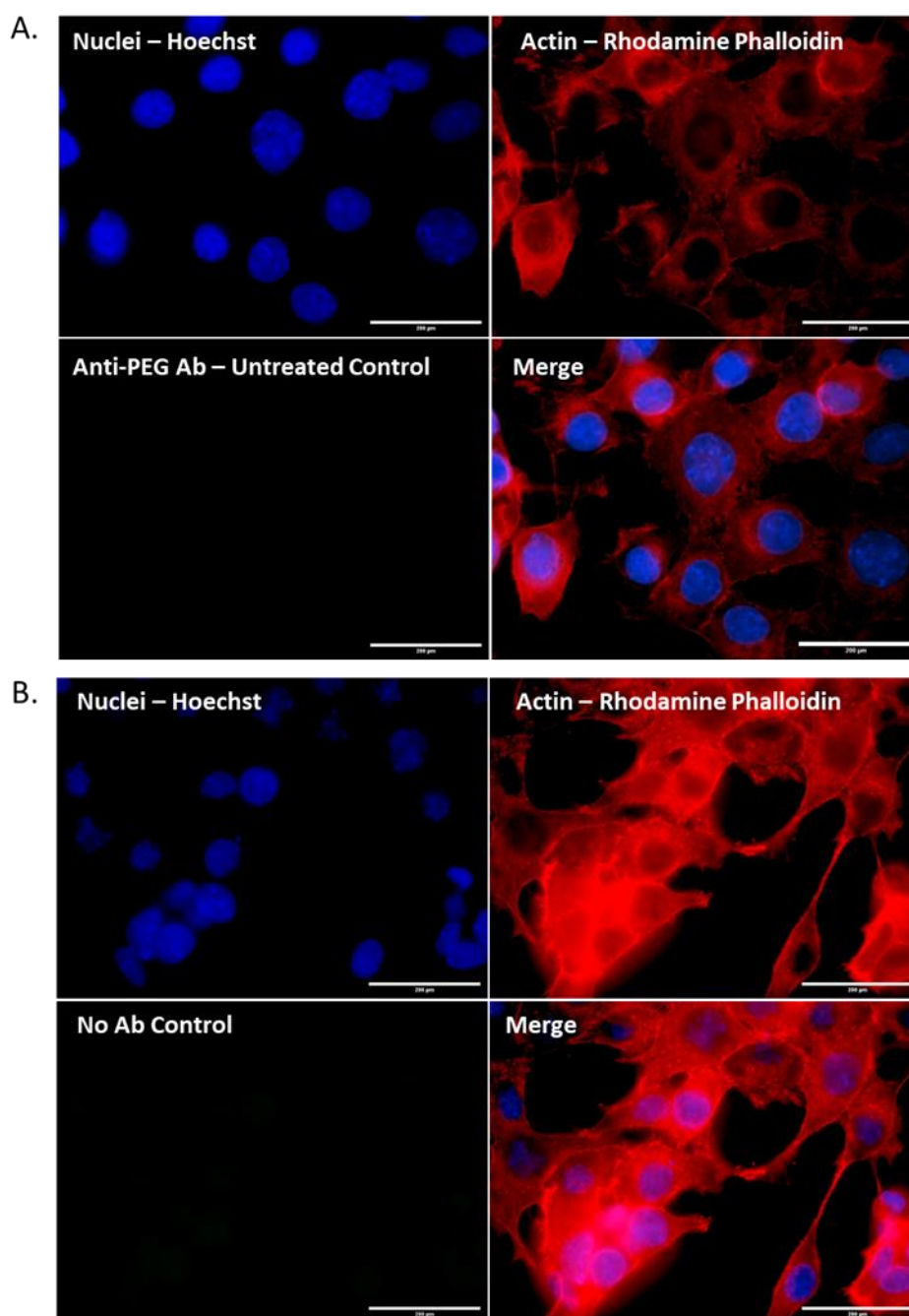


Figure 3.5. Anti-PEG Antibody validation; untreated and no primary antibody controls.

HepG2 monolayers were formed from 10,000 cells per 0.5 ml culture medium and cultured for 24 h before being fixed, permeabilised, blocked and stained. (A) For untreated control, untreated cells were stained with anti-PEG primary Ab overnight, before staining with Alexa Fluor 488 goat anti-mouse secondary antibody (1:500), Hoechst (1:1000) and rhodamine phalloidin (1:25). (B) For no primary Ab control, cells were stained with Hoechst (blue - nuclei) and rhodamine phalloidin (actin – red). Imaging was undertaken using Epifluorescent microscope (Nikon TE300) and 10X objective, with QCapture Software

(QImaging). Images processed using ImageJ Software. No positive staining is observed in either control. Scale bars = 200 μ m.

3.3.2.1 Interaction of AuNP with 2D monolayers of HepG2 cells

HepG2 cells cultured as 2D monolayers were then treated with the first NBM, AuNP, and assessed to determine thresholds readout and cellular morphological and if the NBM interaction could successfully be imaged. 2D cultures were incubated to various concentrations, i.e. 10, 20 and 30 μ g/ml, of the particle for a total time of 24 h to mimic acute exposure conditions.

The interaction between HepG2 monolayers and AuNP was concentration dependent, to varying degrees depending on concentration, with bigger particle clusters observed as concentrations increased. AuNP also appeared to be localised to the cytoplasm, as indicated by white arrows in Figure 3.6.

From this set of experiments, imaging recording, thresholds and NBM interaction and exposure were investigated, optimised and established where to investigate the comparative biological relevance assessment with more complex and challenging HepG2 spheroids.

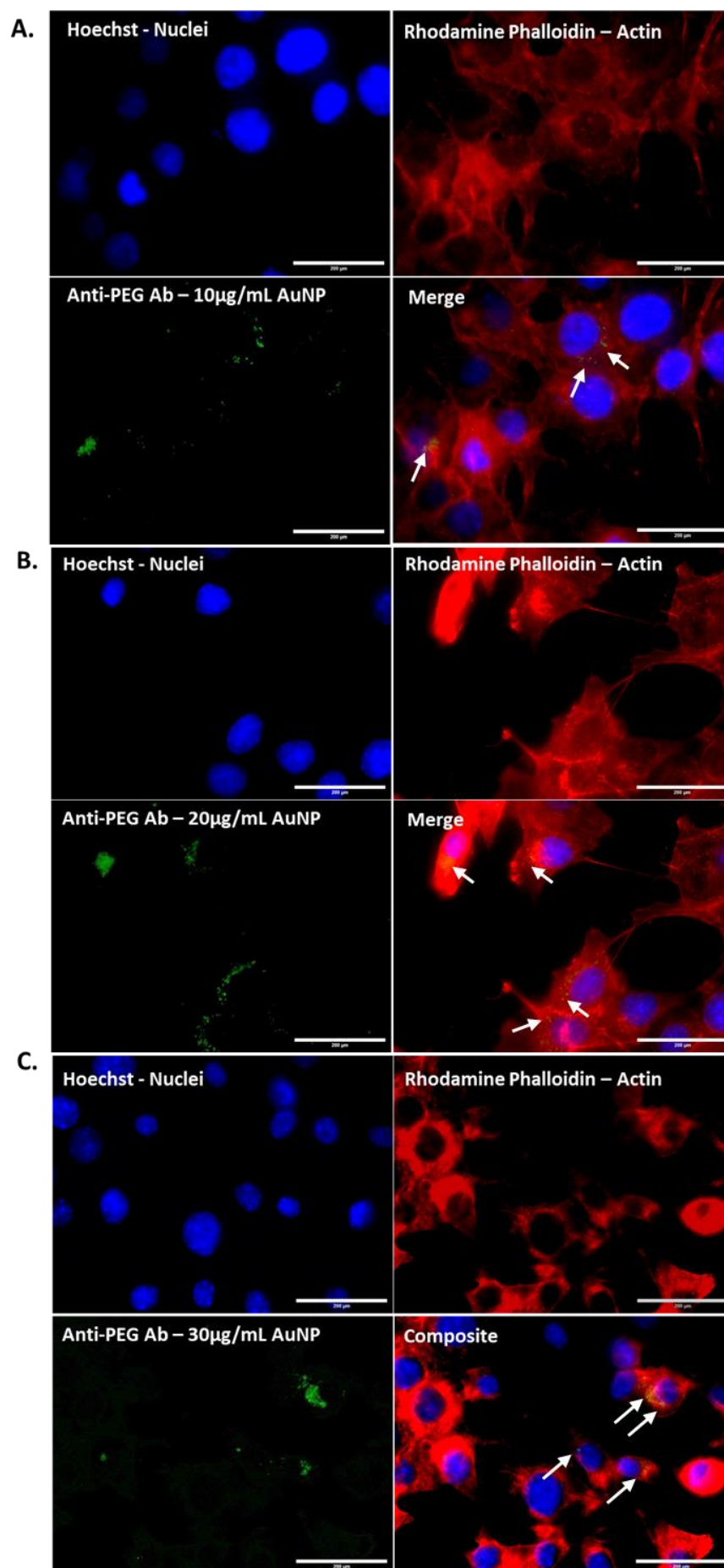


Figure 3.6. Assessment of the intracellular interaction of 20nm PEGylated AuNP in HepG2 2D monolayers.

HepG2 monolayers were formed from 10,000 cells per 0.5 ml culture medium and cultured for 24 h. Representative images of HepG2 monolayers treated for 24 h with (A) 10 µg/ml, (B) 20 µg/ml and (C) 30 µg/ml AuNP. Monolayers were fixed, permeabilised and blocked before being stained with anti-PEG primary Ab overnight, followed by goat anti-mouse 488 secondary Ab (green – PEG), Hoechst (nuclei - blue) and rhodamine phalloidin (actin - red). Imaging was undertaken using Epifluorescence microscope (Nikon TE300) and 10X objective, with QCapture Software (QImaging). n=3 experiments undertaken. White arrows denote particle accumulation in HepG2 cells. All image processing and measurements was undertaken using ImageJ software. Scale bar = 200 µm.

3.3.2.2 Interaction of LipImage in 2D monolayers of HepG2 cells

After the AuNP NBM to 2D monolayer investigation and setting up of experimental conditions, interaction of LipImage NBM with monolayer HepG2 cells was undertaken. Cells were treated with 100, 200 and 500 µg/ml particle for 24 h exposure. From qualitative assessment and a similar manner to the AuNP exposures, HepG2 monolayers internalised LipImage in a density dependent manner. LipImage (green channel) appears to be localised to the cytoplasm, as illustrated in Figure 3.7 and denoted by white arrows.

Again, by undertaking this set of experiments, imaging recording, thresholds and interaction with LipImage was investigated, optimised and established where to investigate the comparative biological relevance assessment with more complex and challenging HepG2 spheroids.

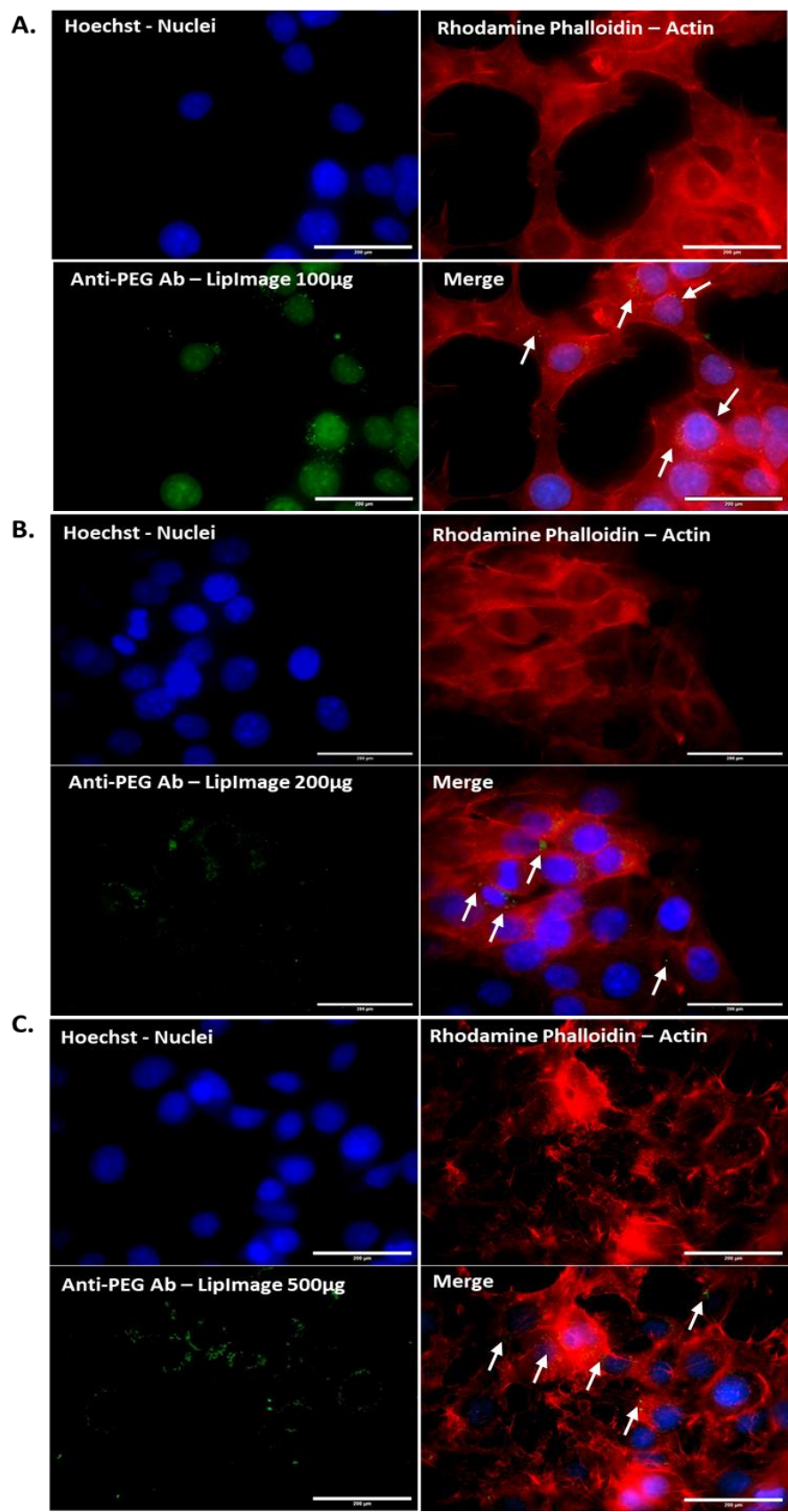


Figure 3.7. Assessment of the intracellular interaction of LipImage in HepG2 monolayers.

HepG2 monolayers were formed from 10,000 cells per 0.5 ml culture medium and cultured for 24 h. HepG2 monolayers were treated for 24 h with (A) 100 µg/ml, (B) 200 µg/ml and (C) 500 µg/ml LipImage. Monolayers were fixed, permeabilised and blocked before being stained with anti-PEG primary Ab overnight, followed by goat anti-mouse 488 secondary Ab (green – PEG), Hoechst (nuclei - blue) and rhodamine phalloidin (actin - red). Imaging was undertaken using Epifluorescent microscope (Nikon TE300) and 10X objective, with QCapture Software (QImaging). n=3 experiments undertaken. White arrows denote particle accumulation in HepG2 cells. All image processing and measurements were undertaken using ImageJ software. Scale bar = 200 µm.

3.3.2.3 Interaction of PACA with 2D monolayers of HepG2 cells

The interaction of PACA NBM was assessed in 2D monolayers. As PACA was also a PEGylated particle, the cell monolayers were stained with anti-PEG Ab in the same manner as AuNP and LipImage. Cells were treated with 10, 20 and 30 µg/ml particle for 24 h exposure. Against expectations, as a reduced amount of PEG is used in the PACA formulation, limited signal was detected in the green channel. PACA however could be detected in both CY5 and the red (TRITC) channels, due to the presence of encapsulated NR668 fluorescent dye.

From qualitative assessment and the same manner to what was observed for AuNP and LipImage exposures, HepG2 cells took up PACA in a concentration dependent manner (Figure 3.8 A, B and C), with particle localised to the cytoplasm, as denoted by zoomed in portion of image and white arrows. Figure 3.8 C, the 30 µg/ml exposure internalized the most PACA.

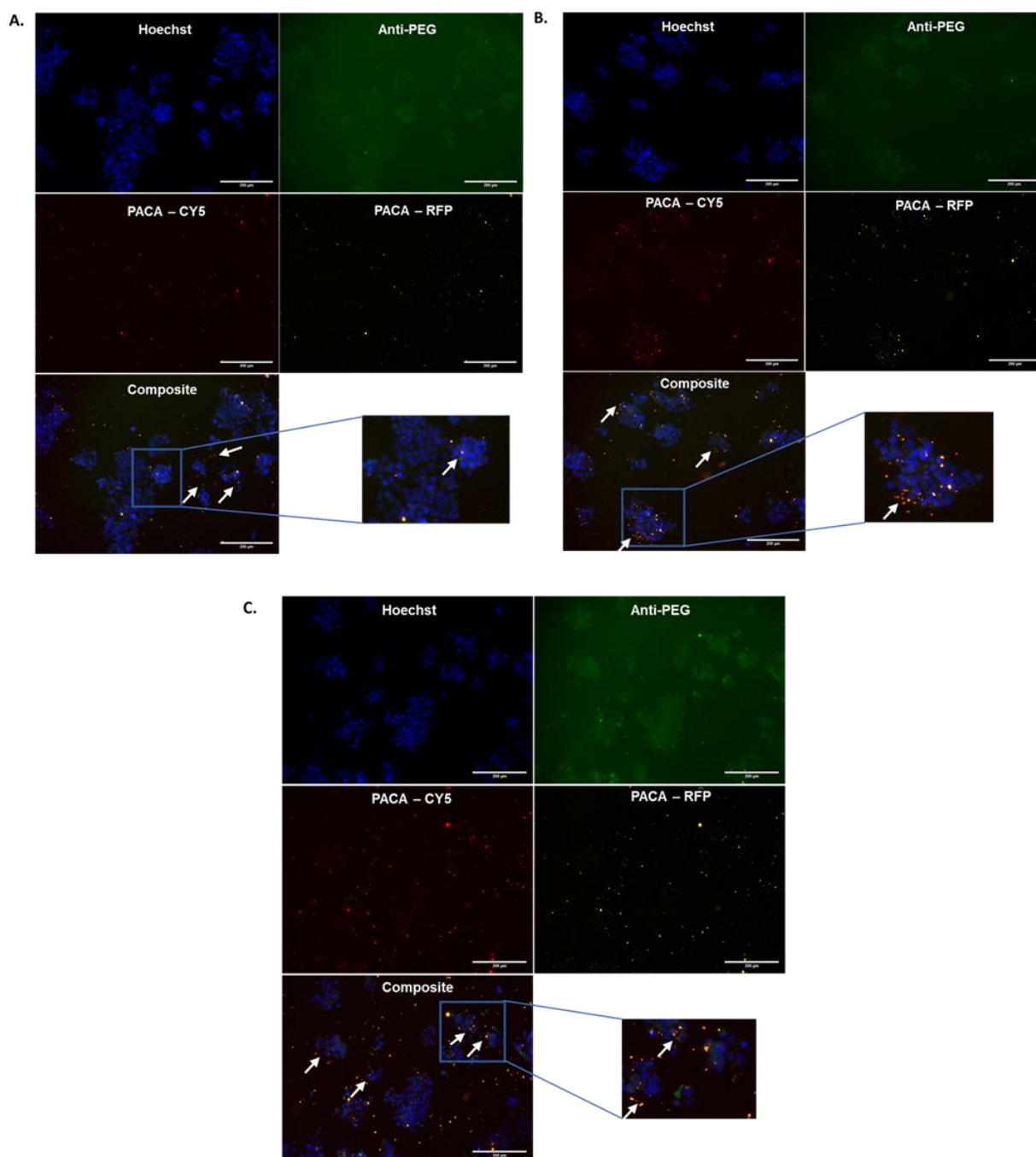


Figure 3.8. Assessment of the intracellular interaction of NR668-loaded polymeric PACA in HepG2 monolayers.

HepG2 monolayers were formed from 10,000 cells per 0.5 ml culture medium and cultured for 24 h. HepG2 monolayers treated for 24 h with (A) 10 µg/ml, (B) 20 µg/ml and (C) 30 µg/ml PACA. Monolayers were fixed, permeabilised and blocked before being stained with anti-PEG primary Ab overnight, followed by goat anti-mouse 488 secondary Ab (PEG - green), and Hoechst (nuclei - blue). n=3 experiments undertaken. Imaging was undertaken using an automated microscope (Lionheart FX, BioTek) and 10X objective, with QCapture

Software (QImaging). Interaction of PACA with HepG2 cells denoted by white arrows. Blue box denoted zoomed portion of image. All image processing and measurements was undertaken using ImageJ software. Scale bar = 200 μ m.

3.3.3 Interaction of NBMs with 3D HepG2 spheroids

Once it was determined that each of the NBMs in this study could be successfully observed, imaged and recorded after exposure in HepG2 2D monolayer cultures, the ability of HepG2 spheroids to interact with the panel of NBMs was assessed. A hepatic spheroid model was chosen as the suitable model for this assessment study due to the pivotal role the liver plays with regard to NBM clearance. Since 99% of I.V. injected NBMs is cleared by the liver, the safety profile of NBM has to be investigated as liver off target side effects and hepatotoxicity. Spheroids cultured for 8 days were incubated with the same increasing concentrations of NBMs as presented for 2D monolayers for a total exposure of 24 h, before being fixed, permeabilised, blocked and stained similarly to the presented 2D monolayers.

3.3.3.1 Assessment of the interaction of AuNPs with 3D HepG2 spheroids

AuNP could successfully penetrate through the outer layer of the dense HepG2 spheroid and accumulate within the spheroids successfully in a dose dependant manner, as presented in Figure 3.9 (AuNP appearing as the green channel). Following exposures to 10 and 20 μ g/ml AuNP (Figure 3.9 A and B), AuNP appears to be localised to the compact regions on the periphery of spheroid (denoted by white arrows), with greater penetration to the spheroid core observed as NBM concentration increases, due to an overall increase in NBM concentration. At the highest concentration, 30 μ g/ml (Figure 3.9 C), AuNP is located throughout spheroid, as indicated in the merged (composite) image, where white arrows indicate areas of spheroid where AuNPs are interacting with HepG2 cells.

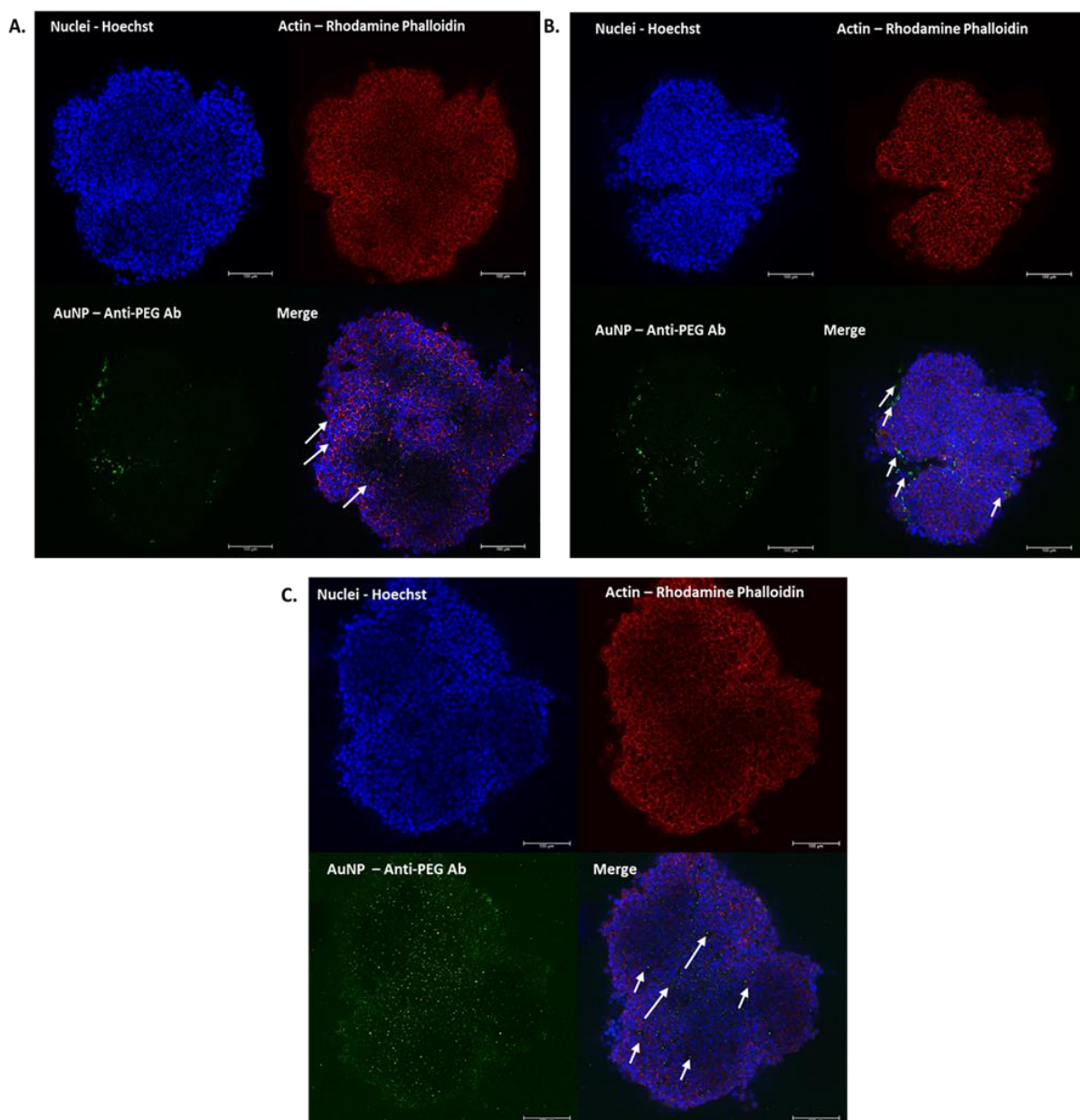


Figure 3.9. Assessment of the intracellular distribution of 20nm PEG-AuNP in HepG2 spheroids.

HepG2 spheroids were formed from 1000 cells in 100 µl culture medium and cultured for 8 d before being treated with (A) 10 µg/ml, (B) 20 µg/ml and (C) 30 µg/ml AuNP. Spheroids were washed, fixed, permeabilised and blocked before being stained with anti-PEG primary Ab overnight, followed by goat anti-mouse 488 secondary Ab (PEG - green), Hoechst (nuclei - blue) and rhodamine phalloidin (actin - red). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, Trinity College Dublin, using a Leica SP8 confocal microscope equipped with a 20x dry objective.

Image processing undertaken using LAS X software. Results representative of n=2 experiments. Each channel is presented separately, with NBM observed in green channel. White arrows in merge images (i.e. a composite of all channels) indicate areas of spheroid where AuNPs are interacting with HepG2 cells. Scale bars = 100 μ m.

3.3.3.2 Assessment of the interaction of LipImage with 3D HepG2 spheroids

In light of the optimisation presented for the highly traceable inorganic AuNP NBM, HepG2 spheroids were then incubated and exposed to LipImage, the IR780 dye-loaded liposome NBM. LipImage, appearing as the green channel in Figure 3.10, was observed to successfully penetrate the outer layer of the dense HepG2 spheroids (Figure 3.10 A, B and C), in a similar manner to what was observed previously for the AuNP NBM. Following 100 μ g/ml LipImage exposure (Figure 3.10 A), LipImage is localised to the periphery of the spheroid, as is denoted by white arrows. Increased interaction between LipImage and the HepG2 spheroid is observed following exposures to the two greater concentrations, 200 and 500 μ g/ml (Figure 3.10 B and C), indicating that LipImage is internalized into spheroids in a dose dependant manner. White arrows in Figure 3.10 B and C indicate areas where LipImage has reached spheroid core. Overall, LipImage appears to be localised to the cytoplasm, colocalized with actin staining (Figure 3.10).

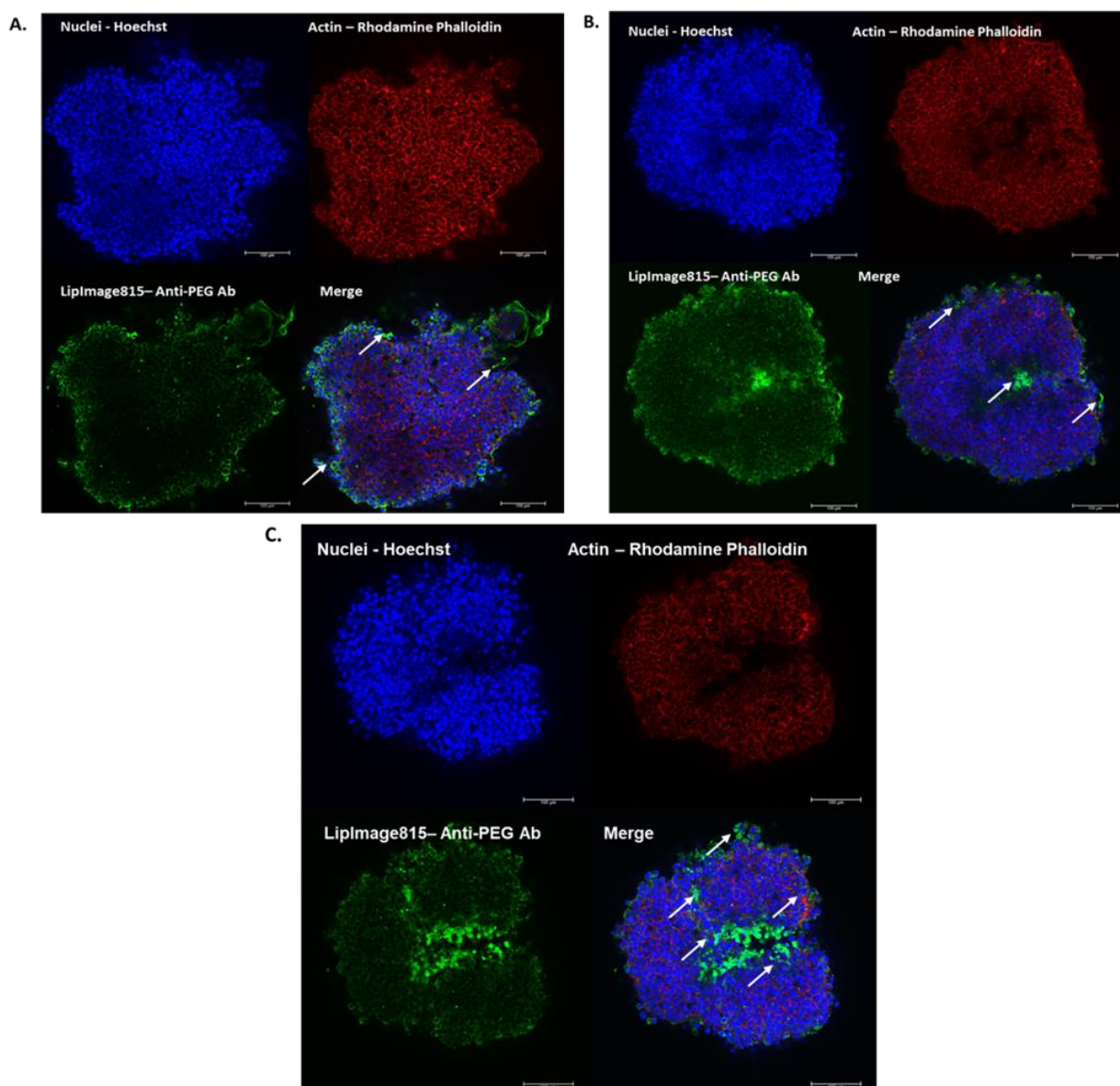


Figure 3.10. Assessment of the intracellular distribution of 20nm LipImage in HepG2 spheroids.

HepG2 spheroids were formed from 1000 cells in 100 μ l culture medium and cultured for 8 d before being treated with (A) 100 μ g/ml, (B) 200 μ g/ml and (C) 500 μ g/ml LipImage. Spheroids were washed, fixed, permeabilised and blocked before being stained with anti-PEG primary Ab overnight, followed by goat anti-mouse 488 secondary Ab (PEG - green), Hoechst (nuclei - blue) and rhodamine phalloidin (actin - red). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, Trinity College Dublin, using a Leica SP8 confocal microscope equipped with a 20x dry objective. Image processing undertaken using LAS X software. Z-stack 100 steps, Total exposures: (300 channels, 100 frames). Results representative of n=2 experiments.

Each channel is presented separately, with NBM observed in green channel. White arrows in merge images (i.e. a composite of all channels) indicate areas of spheroid where LipImage is interacting with HepG2 cells. Scale bars = 100 μ m.

3.3.3.3 Assessment of the interaction of PACA with 3D HepG2 spheroids

The interaction of HepG2 spheroids with the third and final NBM, the NR668-loaded PACA NBM, was assessed. HepG2 spheroids were incubated and exposed to increasing concentrations, namely 10, 20 and 30 μ g/ml PACA, which appears in the CY5 (red) channel in Figure 3.11. Increasing amounts of PACA are visible in HepG2 spheroids as concentration increases, suggesting accumulation occurs in a dose-dependent manner (images representative of n=2 experiments). At the lowest concentration, PACA is localised mostly to the spheroid periphery, as indicated by white arrows in Figure 3.11 A, however as concentration increases PACA is seen to have successfully internalised into the spheroid core, in the tight intercellular spaces between adjacent nuclei (as indicated by white arrows, Figure 3.11 B and C, following exposure to 20 and 30 μ g/ml PACA).

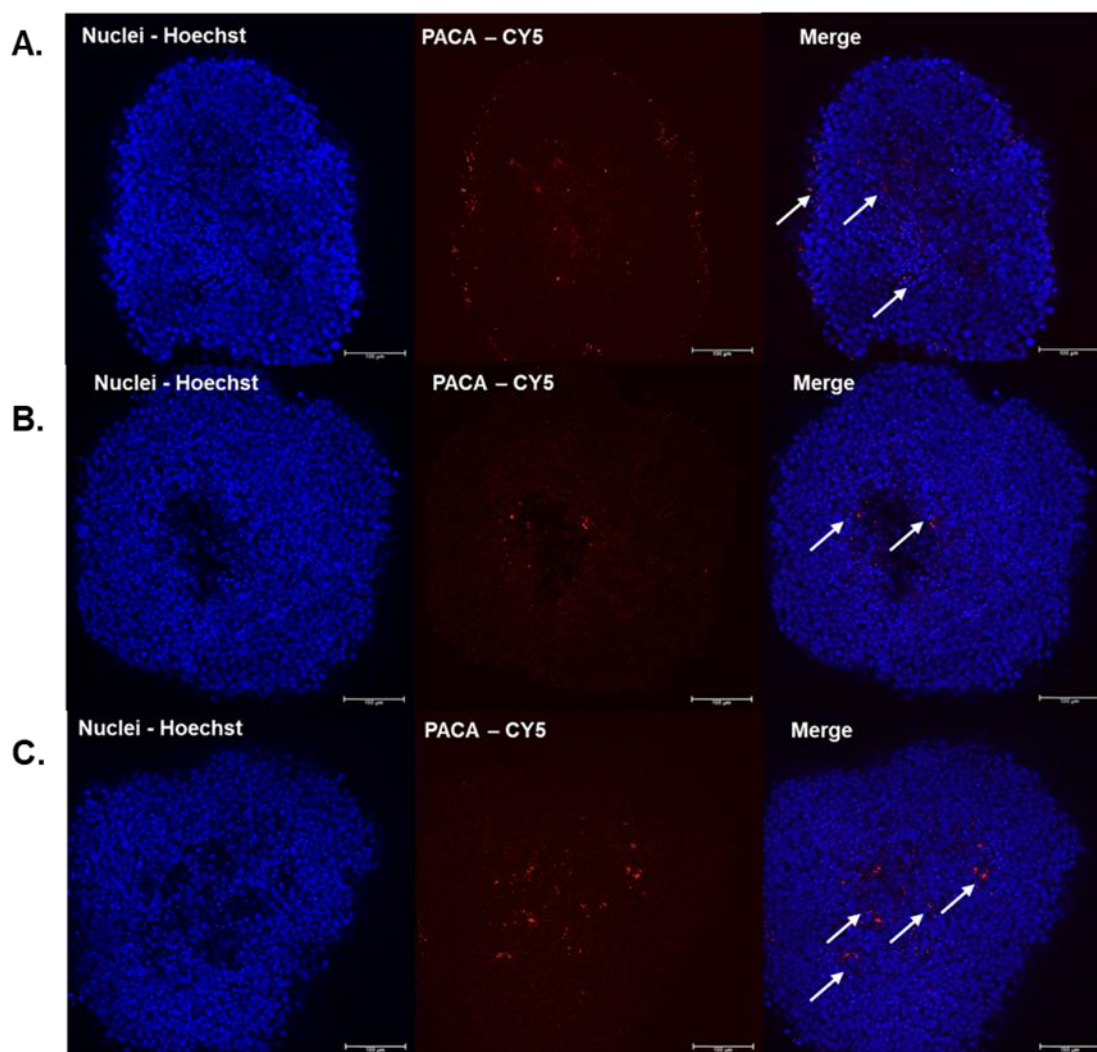


Figure 3.11. Assessment of the intracellular distribution of PACA in HepG2 spheroids.

HepG2 spheroids were formed from 1000 cells in 100 μ l culture medium and cultured for 8 d before being treated with (A) 10 μ g/ml, (B) 20 μ g/ml and (C) 50 μ g/ml PACA. Spheroids were washed, fixed, permeabilised and blocked before being stained with Hoechst (nuclei). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, Trinity College Dublin, using a Leica SP8 confocal microscope equipped with a 20x dry objective. Image processing undertaken using LAS X software. -stack 100 steps, Total exposures: (300 channels, 100 frames). Results representative of n=2 experiments. Each channel is presented separately, with NBM observed in green channel. White arrows in merge images (i.e. a composite of all channels) indicate areas of spheroid where PACA is interacting with HepG2 cells. Scale bars = 100 μ m.

3.3.4 Assessment of the interaction of NBMs with 3D multicellular spheroids of HepG2 and LX-2 cells

Once it was determined that each of the NBMs in this study could be successfully observed, imaged and recorded after exposure in HepG2 3D monoculture spheroids, the ability of HepG2/LX-2 multicellular spheroids to interact with the panel of NBMs was qualitatively assessed.

Multicellular spheroids were cultured for 8 days before being exposed to the largest concentrations of NBMs as presented for 3D monoculture spheroids, namely 30 µg/ml for AuNP and PACA, 500 µg/ml for LipImage, for a total exposure of 24 h, before being fixed, permeabilised, blocked, stained and imaged in the same manner as presented previously for the HepG2 monoculture spheroids (Section 3.3.3).

3.3.4.1 Assessment of the interaction of AuNP, LipImage and PACA with 3D HepG2/LX-2 multicellular spheroids

Each of the panel of NBMs did successfully internalise into the multicellular spheroids, however to a different degree to what was observed with the HepG2 monoculture spheroids (Section 3.3.3). In comparison to what was observed following HepG2 monoculture spheroid exposure to the same concentration of NBM (Figures 3.9 and Figure 3.10 C), AuNP and LipImage both remained on the periphery in the HepG2/LX-2 multicellular spheroids and were not able to penetrate far enough to reach the spheroid core, as denoted by the white arrows in Figure 3.12 A and B. This is in comparison to the HepG2 monoculture spheroids, whereby the largest concentration of both AuNP and LipImage did in fact reach the spheroid core (Figure 3.9 and 3.10 C). As observed in Figure 3.13, a 30 µg/ml exposure to PACA did in fact reach the spheroid core but to a lesser extent than observed in HepG2 spheroids alone (Figure 3.11 C), with the majority of the particle remaining on the spheroid periphery, as denoted by white arrows.

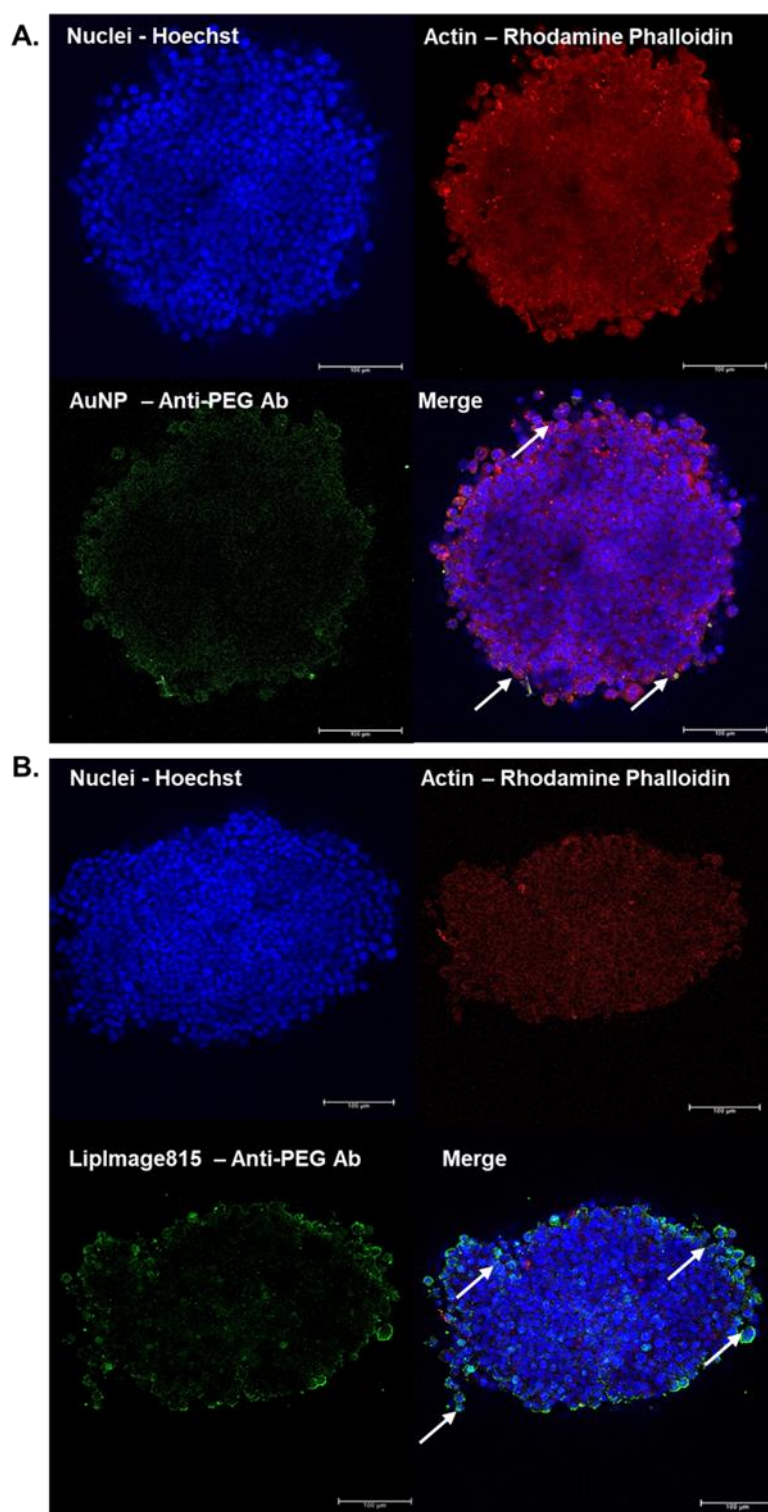


Figure 3.12. Assessment of the intracellular distribution of AuNP and LipImage in HepG2/LX-2 multicellular spheroids (Comparison to Figures 3.09 and 3.10 C).

HepG2/LX-2 multicellular spheroids were formed from 1000 cells in 100 μl culture medium and cultured for 8 d before being treated with (A) 30 μg/ml AuNP and (B) 500 μg/ml LipImage for 24 h. Spheroids were washed, fixed, permeabilised and blocked before being stained with anti-PEG primary Ab overnight, followed by goat anti-mouse 488 secondary Ab (PEG - green), hoechst (nuclei - blue) and rhodamine phalloidin (actin - red).

Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, Trinity College Dublin, using a Leica SP8 confocal microscope with a 20x dry objective. Image processing undertaken using LAS X software. Z-stack 100 steps, Total exposures: (300 channels, 100 frames). Results representative of $n=2$ experiments. Each channel is presented separately, with NBM observed in green channel. White arrows in merge images (i.e. a composite of all channels) indicate areas of spheroid where both AuNP and LipImage are successfully interacting with cells in the HepG2/LX-2 multicellular spheroids. Scale bars = 100 μm .

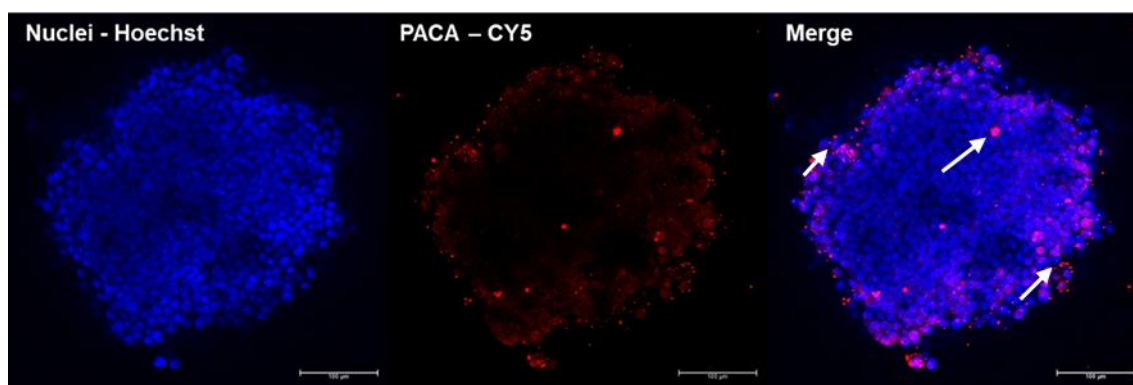


Figure 3.13. Assessment of the intracellular distribution of PACA in HepG2/LX-2 multicellular spheroids (Comparison to Figure 3.11 C).

HepG2/LX-2 multicellular spheroids were formed from 1000 cells in 100 μl culture medium and were cultured for 8 d before being treated with 30 $\mu\text{g/ml}$ PACA for 24 h. Spheroids were washed, fixed, permeabilised and blocked before being stained with Hoechst (nuclei - blue). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, Trinity College Dublin, a Leica SP8 confocal microscope with a 20x dry objective. Image processing undertaken using LAS X software. Z-stack 100 steps, Total exposures: (300 channels, 100 frames). Results representative of $n=2$ experiments. Each channel is presented separately, with NBM observed in green channel. White arrows in merge images (i.e. a composite of all channels) indicate areas of spheroid where both AuNP and LipImage are successfully interacting with cells in the HepG2/LX-2 multicellular spheroids. Scale bars = 100 μm .

3.3.5 Qualitative assessment of the interaction of NBMs with HepG2 monoculture and multicellular spheroids in 3D using 3D reconstruction

In order to gain a more accurate visual representation of the distribution of each NBM in HepG2 monoculture spheroids and HepG2/LX-2 multicellular spheroids, z-stack images for spheroids treated with the highest concentration of each NBM were rendered into 3D images when reconstructed with open-source 3D reconstruction software and bioimaging tool ICY [373]. This approach is useful for qualitative observation since it fully recreates the depth of the 3D environment where NBMs interact with the hepatic cell models.

3.3.5.1 Qualitative assessment of the interaction of AuNP with 3D monoculture HepG2 spheroids and HepG2/LX-2 multicellular spheroids using 3D reconstruction

As a means of gaining an overall clearer view of how AuNP interacted with 3D spheroids and adding further relevance to the z-stack confocal images presented previously in Figure 3.3.3.1 and 3.3.4.1, following the 30 µg/ml AuNP exposure, z stacks were reconstructed using ICY. AuNPs are shown to mostly interaction with cells on the periphery of HepG2 monoculture spheroids, with small amounts of particle visible in the core (Figure 3.14 A) (Single stack confocal image seen in Figure 3.09). In comparison to this, as observed for the HepG2/LX-2 multicellular spheroids (Figure 3.14 B), less NBM is visible at the core due to the more compact nature of the multicellular spheroid (Single stack confocal image seen in Figure 3.12 A).

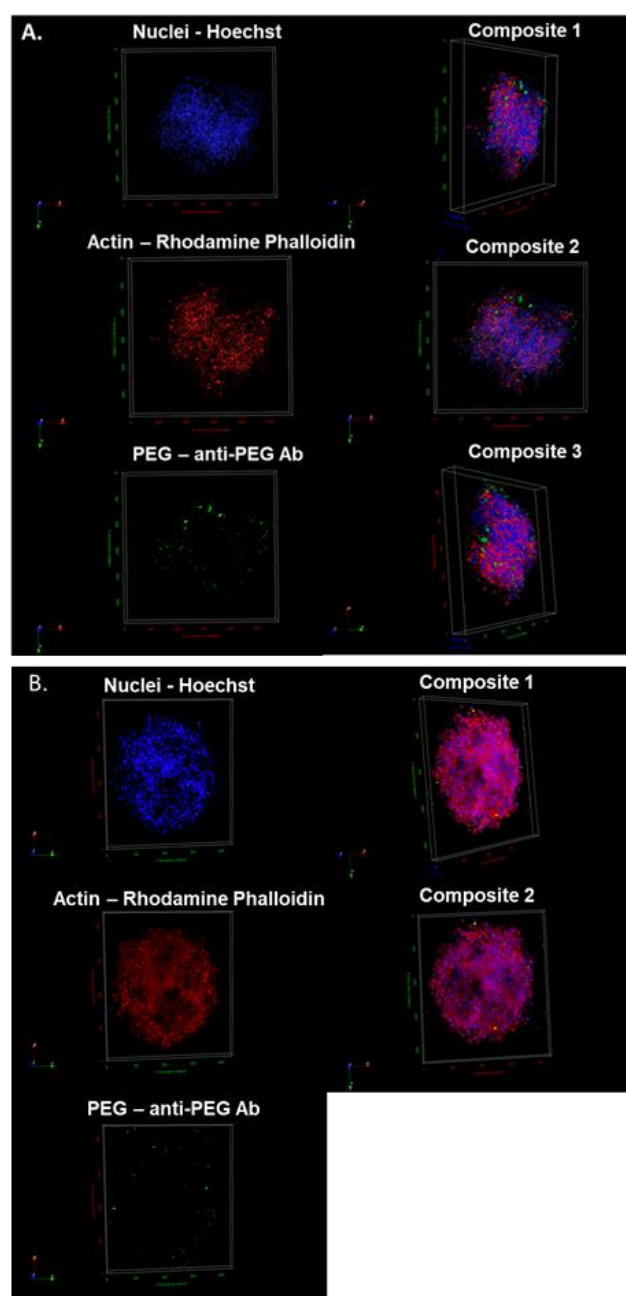


Figure 3.14. Representative images of 3D segmented 3D volumes from HepG2 monoculture and HepG2/LX-2 multicellular spheroid z stacks treated with AuNP. Initial z-stack images (Z-stack 100 steps) acquired using LSCM were reconstructed into virtual a stack; each of the segmented classes was then assigned a pseudo-colour (NBMs in green; nuclei in blue, and actin in red) for representative 3D volume. (A) represents 30 $\mu\text{g/ml}$ AuNP in HepG2 spheroids and (B) 30 $\mu\text{g/ml}$ AuNP in HepG2/LX-2 multicellular spheroids. Icy v (Version 2.0.3.0) (open community platform for bioimage informatics, <http://icy.bioimageanalysis.org/>) software with built in Visualization Toolkit (3DVTk) for 3D computer graphics was used to generate 3D volumes. As observed from the composite

tiles, by reconstructing the z-stack images using 3D reconstruction software, a greater understanding of how AuNPs interact with spheroids can be observed.

3.3.5.2 Qualitative assessment of the interaction of LipImage with 3D monoculture HepG2 spheroids and HepG2/LX-2 multicellular spheroids using 3D reconstruction

To obtain a clearer image of how LipImage interacted when 3D spheroids were exposed to 500 µg/ml this NBM, z stacks were reconstructed using ICY, adding further relevance to the work presented previously in Figure 3.3.3.2 and 3.3.4.2. LipImage is strongly observed in the spheroid core in HepG2 monoculture spheroids, as seen in Figure 3.15 A (Single stack confocal image seen in Figure 3.10). In comparison to this, when multicellular spheroids are exposed to LipImage, the NBM only appears to interact with cells on the spheroid periphery (Figure 3.15 B) (Single stack confocal image seen in Figure 3.12 B).

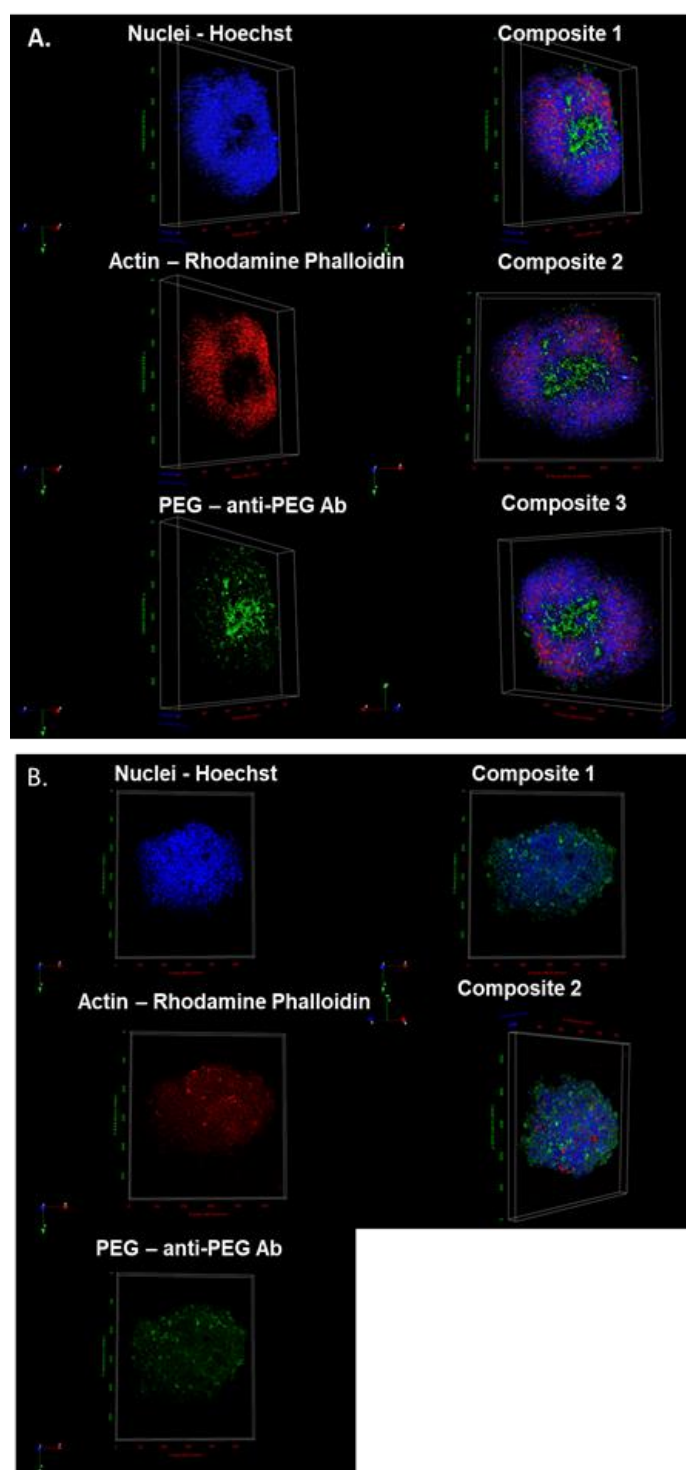


Figure 3.15. Representative images of 3D segmented 3D volumes from HepG2 monoculture and HepG2/LX-2 multicellular spheroid z stacks treated with LipImage. Initial z-stack images (Z-stack 100 steps) acquired using LSCM were reconstructed into virtual a stack; each of the segmented classes was then assigned a pseudo-colour (NBMs in green; nuclei in blue, and actin in red) for representative 3D volume. (A) represents 500 µg/ml LipImage in HepG2 spheroids and (B) 500 µg/ml AuNP in HepG2/LX-2 multicellular spheroids. Icy v (Version 2.0.3.0) (open community platform for bioimage

informatics, <http://icy.bioimageanalysis.org/>) software with built in Visualization Toolkit (3D VTK) for 3D computer graphics was used to generate 3D volumes. As observed from the composite tiles, by reconstructing the z-stack images using 3D reconstruction software, a greater understanding of how LipImage interacts with spheroids can be observed.

3.3.5.3 Qualitative assessment of the interaction of PACA with 3D monoculture HepG2 spheroids and HepG2/LX-2 multicellular spheroids using 3D reconstruction

3D reconstruction was also undertaken on the final NBM, PACA, in order to obtain a clearer image of how a 24 h 30 µg/ml exposure of this NBM interacted with both monoculture and multicellular hepatic 3D spheroids, adding further relevance to the work presented previously in Figure 3.3.3.2 and 3.3.4.2.

PACA is observed to a small degree in the spheroid core, as observed in Figure 3.16 A (red channel). (Single stack confocal image for PACA seen in Figure 3.11). In HepG2/LX-2 multicellular spheroids, PACA appears to mostly interact with cells on the spheroid periphery, with some reaching the core (Figure 3.16 B). (Single stack confocal image seen in Figure 3.13).

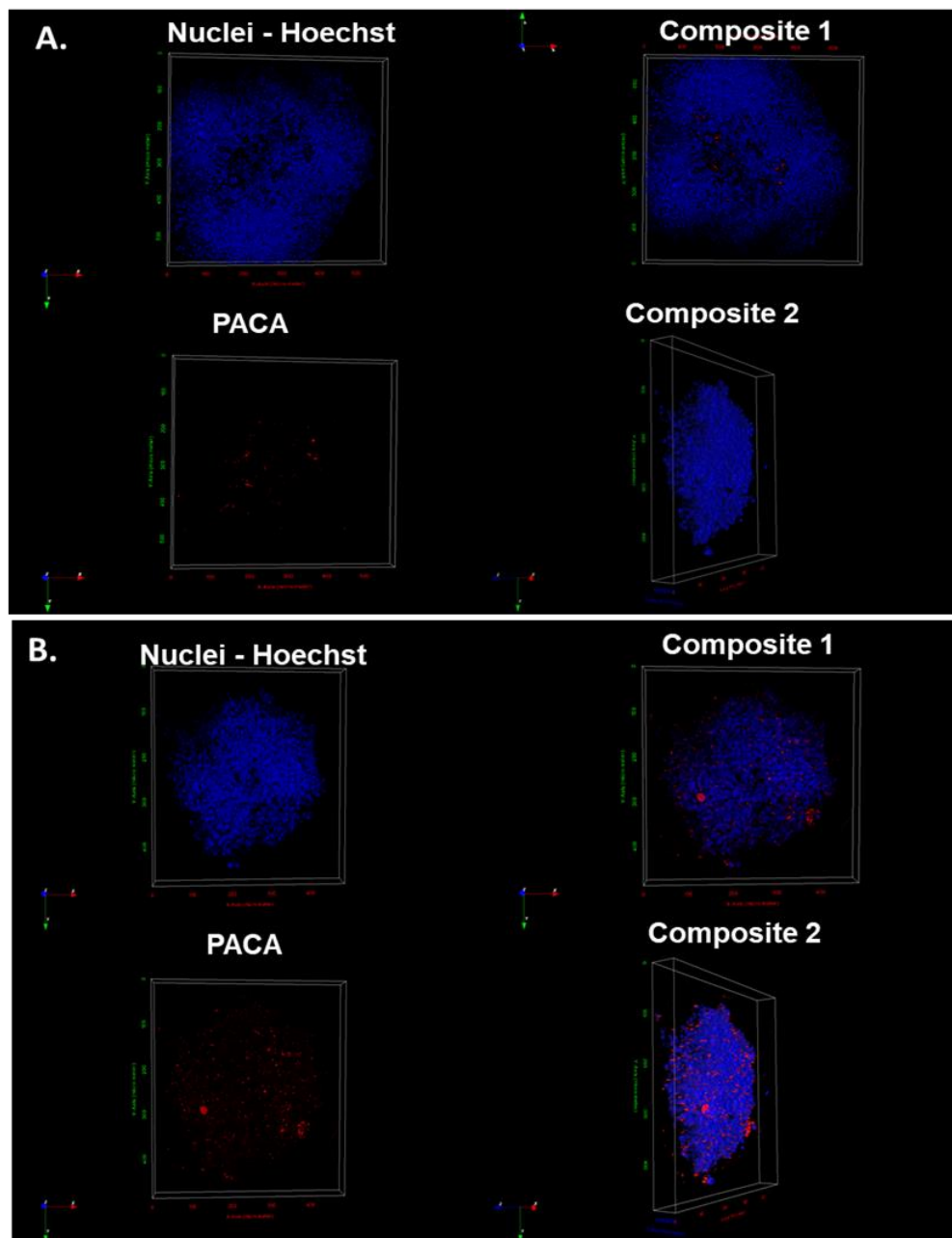


Figure 3.16. Representative images of 3D segmented 3D volumes from HepG2 monoculture and HepG2/LX-2 multicellular spheroid z stacks treated with PACA.

Initial z-stack images (Z-stack 100 steps) acquired using LSCM were reconstructed into virtual a stack; each of the segmented classes was then assigned a pseudo-colour (NBMs in green; nuclei in blue, and actin in red) for representative 3D volume. (A) represents 30 µg/ml PACA in HepG2 spheroids and (B) 30 µg/ml PACA in HepG2/LX-2 multicellular spheroids. Icy v (Version 2.0.3.0) (open community platform for bioimage informatics, <http://icy.bioimageanalysis.org/>) software with built in Visualization Toolkit (3DVTk) for 3D computer graphics was used to generate 3D volumes. As observed from the composite tiles, by reconstructing the z-stack images using 3D reconstruction software, a greater understanding of how PACA interacts with spheroids can be observed.

3.5 Discussion

Following on from Chapter 2, where the ability of the hepatic spheroids to work on their own was assessed, in this chapter their ability to work with a panel of representative NBMs with applications in medical technology was tested, with the overall aim of this chapter to assess the ability of the 3D hepatic monoculture and multicellular spheroids to provide qualitative information on NBM accumulation in the liver that is comparable to what is predicted via *in vivo* studies in animals, i.e. their biological relevance.

Despite the many advantages of NBMs, a significant challenge to using drug or dye carrying NBMs in cancer therapy is their limited penetration in solid tumours and tissues [361]. When discussing the ability of NBMs to successfully enter tissues, the ability NBMs to penetrate dense ECM and be taken in to target cells must be considered [290, 364, 365]. There is therefore a pressing need for the development of *in vitro* models which can accurately assess their interactions with tumours and tissues, beyond the conventional *in vitro* models. Prior to undertaking any *in vitro* experimentation using hepatic spheroids in this thesis, it was vitally important to assess whether HepG2 spheroids could successfully interact with the chosen panel of NBMs, each of these materials representative of a NBM class currently in trials or used in the clinic, and if so, could this interaction be successfully visualised.

In this chapter the *in vivo*-like HepG2 monoculture and multicellular spheroids were treated with three different NBMs, each with varying sizes and physico-chemical characteristics, one benchmark inorganic metallic NBM, and two organic NBMs which encapsulate diagnostic dyes. These three are: an inorganic metal nanoparticles (AuNP); a representative of an organic NBM, an IR780 dye-loaded liposome (LipImage), and a subset of the PACA polymeric NP family, the NR668-loaded PEBCA. As it is widely known that NBM physico-chemical properties play critical roles in their internalization and biodistribution in tissues, physico-chemical characterisation of each of the NBMs was first performed.

The hepatic spheroids were then treated with varying concentrations of each NBM, detailed in Table 3.1, with these concentrations chosen based upon literature and what was noted as relevant from collaboration within the aforementioned REFINE project.

An AuNP was chosen to represent the cohort of metallic NPs. Used as drug carriers, diagnostic agents and tracers, AuNPs can be fine-tuned and synthesized to sizes ranging from 1nm to 100nm, with different charges and a wide variety of coatings, including PEG, citrate, polystyrene, silica, PVP and carboxyl. In this study, a 20 nm PEGylated AuNP NBM was purchased commercially from nanoComposix, with 20nm size chosen as the target size for this study due to the fact AuNPs of this size tend to accumulate in the liver

(predominantly the Kupffer cells) and spleen (approx. 70%) after I.V administration, in comparison to smaller AuNPs (e.g., 1, 2 and 6 nm) which are normally distributed across different organs in the body due to their ultrasmall structures [377], or larger sized AuNPs which may not reach the liver *in vivo*.

To date, a small number of studies have assessed uptake and penetration of various sized AuNPs in spheroids of different cell types, with inconsistencies shown between studies [290, 377, 378]. In general, size plays a critical role in NBM uptake in spheroids, with smaller AuNPs (i.e. 1 – 20 nm) tending to penetrate cell spheroids to a greater extent than their larger counterparts. This penetration also has been shown to increase as incubation time increases, i.e. 3 h to 24 h [290, 377]. As well as NBM size, shape and surface chemistry also plays a large role in uptake and penetration of NBMs in spheroid cultures, with more spherical NBMs achieving more efficient penetration than rod-shaped NBM [378]. HepG2 spheroids have been used by Chelobanov *et al.* to assess penetration of AuNPs, AuPEI, AuBSA NPs. In contrast to other studies, the AuNPs (10 nm) did not penetrate hepatocytes in 2D or as spheroids, and whilst many published studies do show AuNP of this size can in fact penetrate cell spheroids) [379], this data did correspond to other publications that showed using TEM that 10 nm AuNPs did not penetrate hepatocytes in mouse livers, but were taken up into endothelial cells and Kupffer cells [380]. It is also hypothesised that this lack of AuNP uptake in hepatocytes may be due to the presence of serum which contains proteins that form a corona (i.e. the protein corona), thus changing the pattern of AuNP and cellular interaction. With these inconsistencies across literature, it is safe to say that much more research is needed to fully elucidate the factors that influence AuNP uptake into cells and spheroids.

In this study, 2D HepG2 monolayers were the first cultures to be treated with the chosen AuNP. Before assessing the 3D spheroids, a pilot set of control experiments was undertaken to assess the hepatocytes and morphological responses in 2D and subsequently if they could be successfully imaged using an anti-PEG antibody. Exposures on 2D monolayer cultures were undertaken for all NBMs to confirm NBMs could successfully be visualised in monolayers before moving into spheroid cultures. For the AuNP, HepG2 monolayers were exposed to increasing concentrations, i.e. 10, 20 and 30 µg/ml for 24 h, before cells were fixed, permeabilised and stained with anti-PEG antibody for particle visualisation. AuNPs accumulate in HepG2 cells was directly proportional to concentration used, as observed in Figure 3.6. The greatest amount of AuNP and small clusters of AuNPs are observed following the largest exposure, 30 µg/ml. After it was confirmed that monolayers successfully interact with the AuNP and that the anti-PEG antibody was an appropriate way of visualising them (Figure 3.6), HepG2 spheroids were treated with the same

concentrations. AuNPs successfully interact with spheroids, doing so in a concentration dependent manner (Figure 3.9 A, B and C). After treatment with 10 µg/ml, particles were observed localized to the periphery of spheroid (Figure 3.9 A). Greater internalization was observed at the larger concentrations, where particles appeared to localise in the core. This is in contrast to work published by Huang *et al.* who found that 15 nm AuNPs clustered around the surface of spheroids, potentially due to their larger sizes hindering their penetration [377] (Supplementary Figure S3.4), but in accordance with the previously cited study undertaken by Rane and Armani, who illustrated that 30 nm AuNPs had higher uptake in spheroids, reaching larger depths than their 10nm counterparts, who only reached less than half the depth in spheroids as the 30 nm [378].

In comparison to what was observed in HepG2 monoculture spheroids however, far less particle was seen to interact with the multicellular spheroids. Here, AuNP at the largest concentration (30 µg/ml) remained on the spheroid periphery, in comparison to the monoculture spheroids where the same concentration was found to reach the spheroid core (Figure 3.12 A). This is a surprisingly result considered the expectation that incorporating an NPC cell line into the spheroid model who increase interaction of NBMs with the model. It can be hypothesised that the inability of AuNP to successfully penetrate the spheroid core may be due to the LX-2 cells imparting compactness on the spheroids. As detailed in a study by Song *et al.*, the addition of LX-2 cells to HepG2 spheroids significantly increases the compactness of spheroids. In this study, it was determined that the interaction of LX-2 cells with HCC cells imparted strong compacting properties on HCC spheroids, via the accumulation of collagen 1A1 (COL1A1), a factor which in turn facilitated chemoresistance in the spheroids [357]. Pingitore *et al.* have also reported similar findings [302]. Various *in vivo* studies have used 20nm AuNP in animal models. Balasubramaniam *et al.* have shown that 20nm uncoated AuNPs rapidly and consistently accumulate in the livers of rats, with 49.4 ± 50.4 , 64.8 ± 39.7 and 60.3 ± 19.9 ng/g of tissue observed in the liver one day, one week and one month respectively post IV administration [381]. In nude mice bearing subcutaneous A431 squamous tumours, Zhang *et al.* have assessed with biodistribution and pharmacokinetics of various size PEG-AuNPs including 20 nm. Here it was shown that 20nm AuNPs had the highest uptake in the liver throughout the study [382]. Bailly *et al.* have assessed 21nm AuNPs in healthy mice (1mg/kg dose), also showing that AuNP of this size preferentially accumulate in the liver, with more than 35% of injected dose present in the liver after 24 h, and more than 50% present one- and two-weeks post injection. Microscopic analysis showed normal healthy hepatocytes, with no signs of inflammation, steatosis or fibrosis. The subcellular location of the AuNPs was found to be clustered inside

phagocytosing cells in liver (Kupffer cells) however, with no AuNPs detected in hepatocytes or endothelial cells in the liver [368] (Supplementary Figure S3.5). This is in concordance with other studies that suggest AuNP are taken up by hepatocytes [383] (Supplementary Figure S3.6), but consistent with the pharmacokinetic profile detailed in literature for AuNP coated with dextran and not PEG.

Some studies however have also reported that PEG AuNPs are also taken up by Kupffer cells, including a study from Chen *et al.* [384] (Supplementary Figure S3.7). These results demonstrate the importance of relevant cell types in 3D multicellular spheroid models. In this thesis, it was shown that AuNPs can successfully interact with cells in both the monoculture and multicellular hepatic models. The incorporation of a different cell type, with preference of Kupffer cells, would further enhance this study. A pharmacokinetic (PK) *in vivo* animal study which is due to be undertaken in early 2021 will also add great value to the overall understanding of the interactions of this specific AuNP with the cells of the liver and will also greatly add to this study.

Following on from the assessment of the benchmark AuNP, the second NBM assessed in both hepatic monolayers and spheroids was LipImage, an IR780 dye-loaded liposomes used as a near infrared *in vivo* fluorescent imaging tool, and an NBM which to date has not previously been studied in hepatic spheroids, making the work presented here a significant advance for this material. As liposomes are soft colloidal NPs, tumour and tissue penetration generally occurs by means of particle diffusion, through intercellular spaces and cellular uptake [105, 385-389]. Whilst uptake of LipImage has yet to be studied *in vitro* using monolayers or spheroids, some studies have used spheroids to study uptake and distribution of liposomes in general, in spheroids formed from a variety of cell types. In general, it has been shown that liposomes can be taken up by spheroids successfully, with penetration not limited by treatment time but more so the intercellular space present in spheroids [361]. It has also been shown that positively charged liposomes show a large overall binding and affinity for spheroids, irrespective of the particle size [390]. The uptake of the NBM Caelyx[®] (liposomal doxorubicin) has also been compared to free doxorubicin in spheroids and polymeric NBMs, demonstrating that NBM penetration, accumulation and internalization in spheroids was highly dependent on NBM size, with polymeric particles of 50 nm showing greater internalization than their 100 nm counterparts, and with Caelyx[®] (approx. 87 nm) showing penetration somewhat between the polymeric NBMs, with these results suggesting that the extracellular space in spheroids, coupled with the ECM matrix has a size restricting implication for NBM penetration [391]. Zhang *et al.* have used HepG2 spheroids (cultured to 7 d) to assess PEGylated liposome uptake and penetration [291]. Here it was shown by

confocal microscopy that liposomes successfully move through the different layers of the spheroids (Figure 3.17), with unmodified PEGylated liposomes mainly distributed strongly in the spheroid core, in a similar pattern as observed in this study (Figure 3.10).

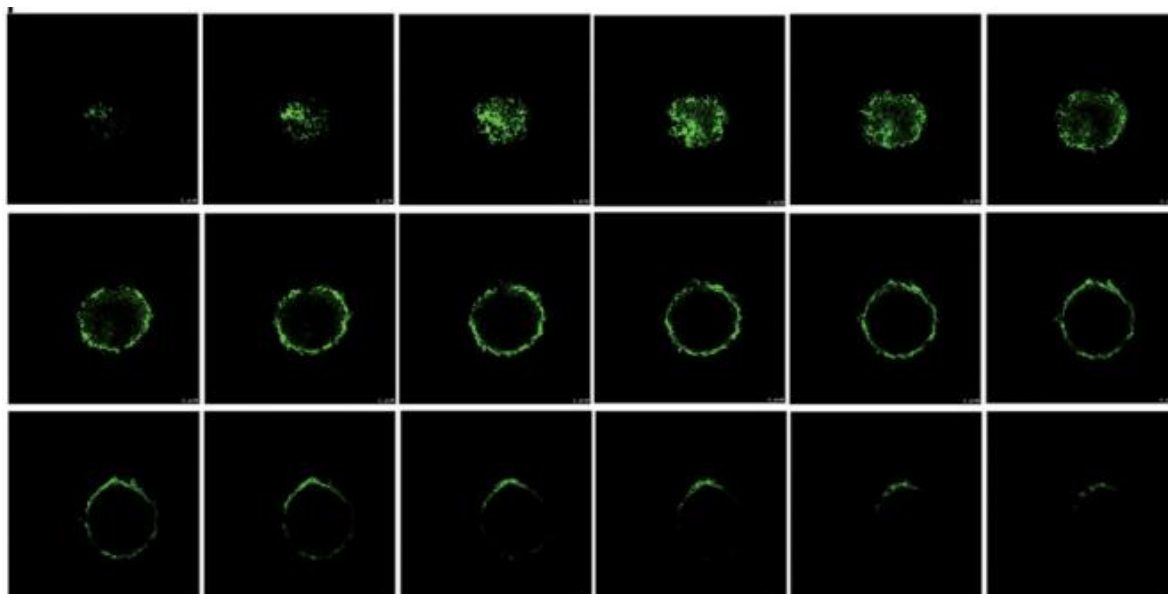


Figure 3.17. Confocal microscopy images with an 8 μm interval between consecutive slides of HepG2 tumour spheroids after incubation with unloaded liposomes.

Time lapse of 20 Scale bar represents 100 μm . Figure reproduced from [291].

As observed in Figure 3.7, LipImage can be observed in HepG2 monolayers at each of the doses chosen (100, 200 and 500 $\mu\text{g/ml}$), with hepatocytes taking up the NBM in a concentration dependent manner, with larger amounts visible at 500 $\mu\text{g/ml}$. This pattern is also mirrored but to a much stronger degree in HepG2 spheroids (Figure 3.10), with LipImage localised in the cytoplasm, also occurring in a concentration dependent manner. Liposomes appear more in the periphery of spheroids at 100 $\mu\text{g/ml}$, with penetration to the core observed for the 200 and 500 $\mu\text{g/ml}$ concentrations. Whilst a direct comparison to literature cannot be made as the work presented in this thesis is the first time LipImage has been studied in such a way, two animal studies has assessed LipImage *in vivo*.

Sayag *et al.* have shown that LipImage strongly fluoresces predominantly in liver of healthy beagle dogs four h post injection, with a significant increase in the liver 1-day post injection of a dose of 102 nmol/kg, and sustained up to 21 days compared to other organs [392] (Figure 3.18). The high accumulation LipImage in the liver, determined from fluorescence imaging, is in accordance with previous mouse studies that also show LipImage is preferentially taken up by the liver [34] (Supplementary Figure S3.8).

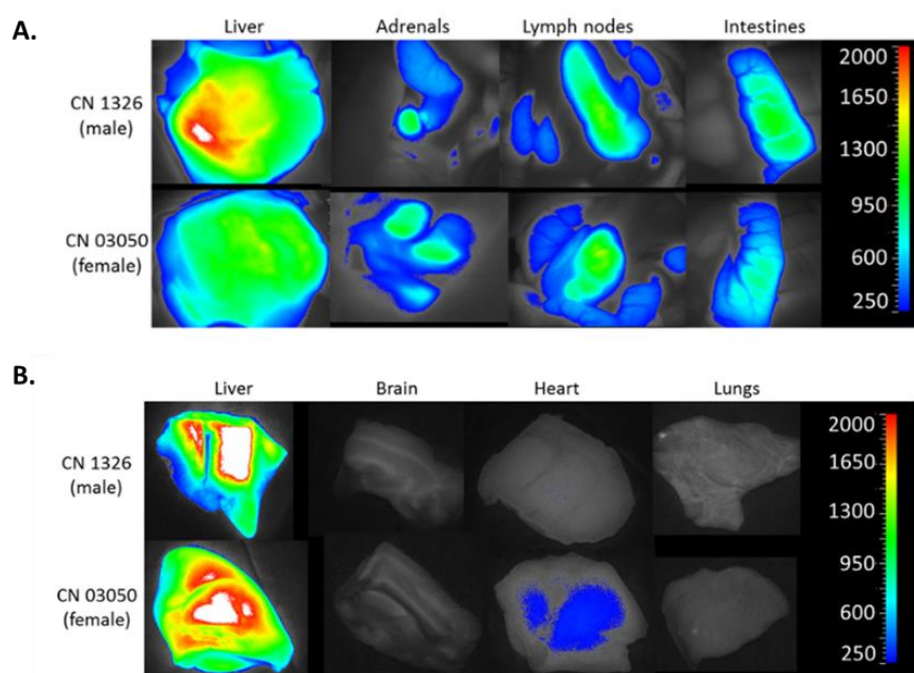


Figure 3.18. Post-mortem fluorescence biodistribution of LipImage™815 in key organs from healthy beagle dogs.

Figure reproduced from [392]. (A) 24 h and (B) 72 h after intravenous injection of LipImage in healthy dogs (group 3, phase 0). The fluorescence contrast was set between 250 and 2000 and black and white image contrast between 0 and 600.

This similar strong fluorescence is not observed in the 2D monolayer 500 µg/ml treatment, thus emphasising the biological relevance of the hepatic spheroid models over 2D monolayer cultures. In a similar manner to what was observed following the AuNP treatment, the largest concentration of LipImage did not successfully penetrate the core of HepG2/LX-2 multicellular spheroids, with particle remaining on the periphery (Figure 3.12 B). It can be hypothesised that this is again due to the increased compactness of the spheroid models when LX-2 cells are incorporated, coupled with the less persistent and the soft colloidal nature of liposomes in general.

Due to the difficult in detecting LipImage on a cellular level, due to the fact that unless tagged with a fluorophore, liposomes in general cannot be detected at this level, no histopathology was undertaken on liver sections from *in vivo* mouse study, and images from H&E stained liver sections from the beagle study were not provided. Unpublished *in vivo* data obtained by the Nanomedicine and Molecular Imaging Group from an animal study

using Balb/C mice however was able to detect LipImage at the cellular level utilising the anti-PEG Ab used in this thesis. No indication of toxicity from histopathological studies, with no necrosis or infiltration of immune cells observed (Figure 3.20). Histopathological analysis also suggests accumulation of LipImage is cytoplasmic, similar to results observed in Figure 3.10.

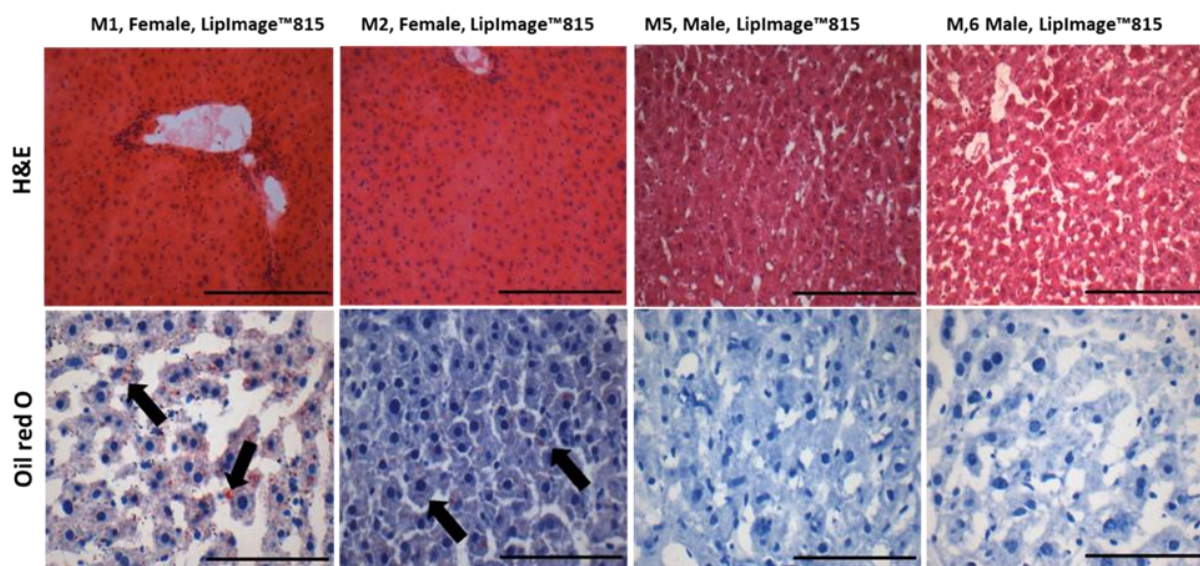


Figure 3.19. Liver sections obtained from an in-house in vivo mouse study of LipImage™815 undertaken at TCD.

Procedures undertaken by Dr. Olivero Gobbo, School of Pharmacy, TCD, and organ processing and staining undertaken by Ms. Anna Bogdanska, Nanomedicine and Molecular Imaging Group, School of Medicine, TCD. Liver samples from mice were cryo-embedded by freezing in OCT in a bath of isopentane at -20°C . Prior to sectioning, samples were stored at -80°C . 20 consecutive sections were cut with a cryotome at $10\mu\text{m}$, and sections were stored at -80°C prior to staining. Consecutive sections were stained in following order: slide 1 H&E for general evaluation of tissue, slide 2 with Oil red O for detection of lipids and following 2 sections kept for any other staining or to repeat any staining if needed. For each organ at least 5 slides were stained with both stains. Staining was performed according to protocols optimised internally in our lab during previous studies. Positive control (omentum) was included with each batch of slides stained with Oil red O. Representative photomicrographs of tissue stained with H&E (top row) or Oil red O (bottom row). Lipid droplets are visible as bright red deposits. Scale bar represents $200\mu\text{m}$ in top images, and $100\mu\text{m}$. Arrows emphasise lipid droplets.

To the best of my knowledge, to date no studies have assessed the interaction of NR668 PEBCA (PACA) in HepG2 monolayers or spheroids. Some studies however have assessed penetration of polymeric NBMs in general in spheroids of varying cell types. In the same study where they compared uptake of free and liposomal doxorubicin, Tchoryk *et al.* also assessed a range of poly(styrene) NBMs, ranging in size from 30 nm to 100 nm and of various surface chemistry, i.e., plain, carboxylated, PEG coated and aminated, and a new biodegradable polymeric particle, PGA [393]. Here, they illustrated that unmodified poly(styrene) NBMs of 30 and 50 nm could successfully penetrate the core spheroids more efficiently than their larger (100 nm) counterparts. This may explain why limited PACA (142 nm in size) was observed in both monoculture and multicellular spheroids (Figures 3.11 and 3.13), in comparison to the large amounts visualised in 2D HepG2 monolayers (Figure 3.8). PGA NBMs of 100 nm demonstrated a similar penetration to 50 nm poly(styrene) NBMs, which is hypothesised to be due to the flexibility of the polymer used to synthesise them. It was also shown in this study that PEGylation significantly improved penetration to the spheroid core [391]. These results are also consistent with results from previous *in vitro* and *in vivo* studies from Tang *et al.* who have shown that the extracellular space and ECM in spheroids have a size restricting characteristic on NBM penetration again, supporting the theory that the extracellular space and ECM have a size restricting characteristic on NBM penetration [363]. PACA could be visualised in each of the three channels, green (anti-PEG Ab), CY5 and RFP, as observed in HepG2 2D monolayers, Figure 3.8. In comparison to the AuNP and LipImage however, only a very faint signal was detected in the green channel, due to the greatly reduced % of PEG on the PACA NBM in comparison to the other two particles. Interaction between PACA and HepG2 monolayers occurred in a concentration dependent manner, with large particle clusters observed 30 µg/ml. The same pattern was observed in HepG2 spheroids (Figure 3.11), occurring in a concentration dependent manner and NBMs appearing to penetrate spheroid core at the largest concentration, 30 µg/ml, with PACA. In multicellular spheroids, PACA did in fact reach the spheroid core, however to a much lesser extent to what was observed in HepG2 monoculture spheroids (Figure 3.13). It can be hypothesised that the ability of PACA to successfully penetrate the core of multicellular spheroids in comparison to AuNP and LipImage may be due to the more persistent nature of polymeric NBMs on a whole. To date, one *in vivo* animal study has been undertaken using this specific PACA NBM. A study in tumour-bearing (MAS98.12) xenograft nude mice has been undertaken on the dye loaded (NR668) and drug and dye loaded (Cabazitaxel (CBZ) + NR668) versions of this NBM [394]. In this study it was shown

that the liver took up the majority of both NR668-loaded and CBZ + NR668-loaded PEBCA, as determined by fluorescence imaging (Figure 3.20).

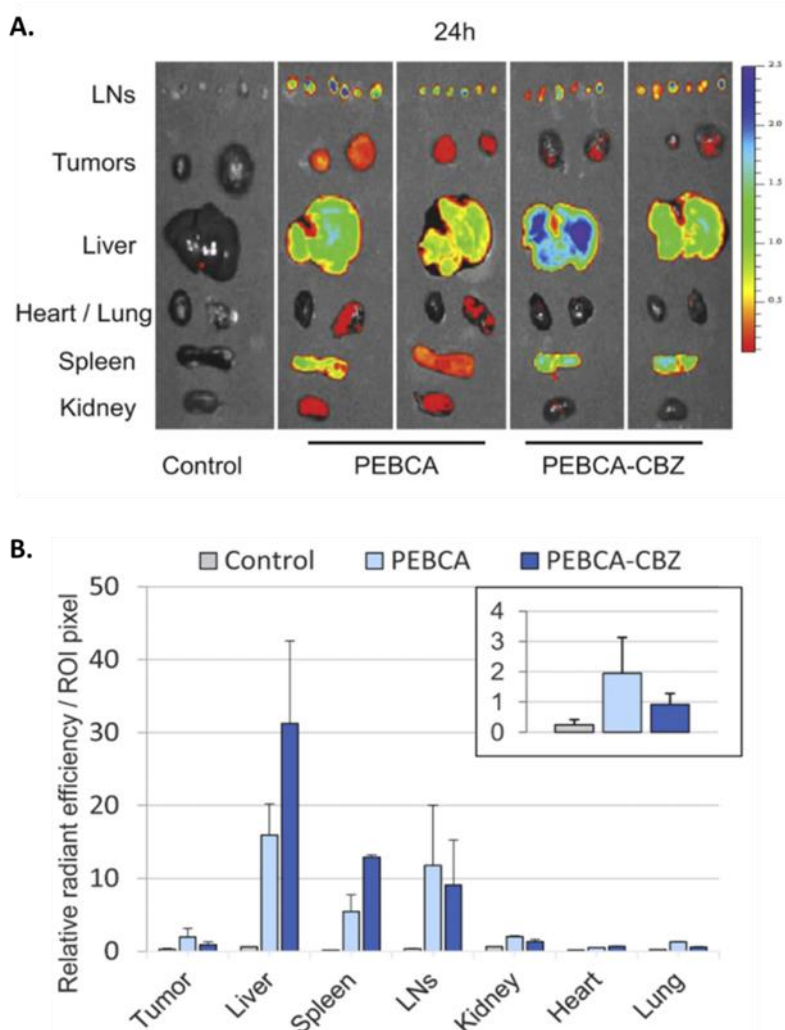


Figure 3.20. Biodistribution of NR668-loaded PEBCA (PACA) and CBZ-loaded PEBCA particles containing the fluorescent dye NR668 measured in MAS98.12 tumour-bearing nude mice.

Figure reproduced from [394], <https://doi.org/10.1016/j.jconrel.2018.11.029>. Whole body images were obtained with IVIS 1, 4, 24 and 96 h after I.V administration of the NBMs; colour scale on the right indicates radiant efficiency $\times 10^9$. (A) Quantification of fluorescence intensity as relative radiant efficiency per region of interest pixel data of tissues collected 24 h after injection. (B). Mean values obtained for 2 animals; error bars show SD values. PEBCA-CBZ: PEBCA containing CBZ and NR668; PEBCA: PEBCA containing NR668, but not CBZ. LNs: lymph nodes. The tumour data are enlarged in the inset.

In contrast to the strong fluorescence observed in spheroids for LipImage treatment, something which corresponded greatly to what is observed *in vivo*, fluorescence intensity of PACA in monoculture and multicellular spheroids was not as strong. A study from Park *et al.* has shown that polymeric NBMs are predominantly taken up by Kupffer cells, with only 7% taken up by hepatocytes [395]. Therefore, it can be hypothesised that the incorporation of KCs into the spheroid model would make a model more predictive of PACA accumulation. A pharmacokinetic (PK) *in vivo* animal study which is due to be undertaken in early 2021 will add great value to the overall understanding of the interactions of NR668-loaded PEBCA (PACA) with the liver and will also add to this study.

The aim here was to determine the biological relevance of hepatic spheroids in predicting how NBMs may accumulate in the liver, and to determine if this predictiveness is comparable and consistent to *in vivo* studies. Predicting this information *in vitro* is vitally important, however to date the current methodologies for assessing NBM penetration and cellular internalization is based upon either greatly reductionist 2D *in vitro* cell cultures or expensive, time-consuming and unethical animal studies. Advanced *in vitro* methodologies such as 3D spheroids have emerged as a potential means of overcoming these issues, however to date their uses in predicting NBM accumulation and uptake in the liver is not widespread, making the results presented in this chapter novel, relevant, and a significant advance to the state of the art for each of these NBMs and hepatic spheroids, and especially with regard to both LipImage and PACA who, up until now, had not been assessed in liver spheroids at all. The hepatic spheroids did act as good predictors of NBM accumulation and interaction *in vivo*, which was the primary aim of this chapter, something which was most largely noted using LipImage. As the confocal images presented in this study are only representative of a single z-stack, it was also shown that 3D rendering could be used as an additional tool for visualising NBMs in spheroids. By reconstructing all stacks of a z-stacks in ICY 3D reconstruction software, it was possible to gain more accurate visual representation of the full depth of NBM distribution throughout the 3D environment and the interaction of NBMs with the cell models. The results presented in this chapter are novel, relevant and a significant advance in the area of using hepatic spheroids to assess NBM interaction and accumulation.

3.6 Conclusion

- Each NBM, i.e. a liposome, a polymeric and a metallic nanoparticle, successfully internalizes and interacts with HepG2 cells cultured as both monolayers and 3D monoculture and multicellular spheroids, to varying degrees and in a concentration dependent manner, as observed from epifluorescent and confocal microscopy.
- Multicellular HepG2/LX-2 spheroids do not exhibit the same internalization as monoculture HepG2 spheroids, potentially due to the more compact nature of these spheroids.
- Using 3D rendering and reconstruction software is a useful method of visualising z-stack confocal images of treated spheroids in a more representative 3D manner, allowing a greater view of NBM distribution throughout spheroids.
- As each particle was shown to successfully internalize and interact with spheroids, penetrating the dense cell layers, it was determined that the spheroids could be carried forward and used for the *in vitro* assessment of each of the NBMs, which will be discussed in the following chapter.

Chapter 4

Assessing the hepatotoxicity of NBMs using 3D hepatic spheroid models

Chapter 4 - Assessing the liver toxicity of NBMs using 3D hepatic spheroid models

Aim

To determine the relevance of 3D hepatic spheroid models developed for assessing NBM liver toxicity. This refers to the ability of 3D models to represent a suitable *in vitro* model which exhibit cytotoxicity and genotoxicity profiles comparable to that detected *in vivo* animal studies.

4.1 Introduction

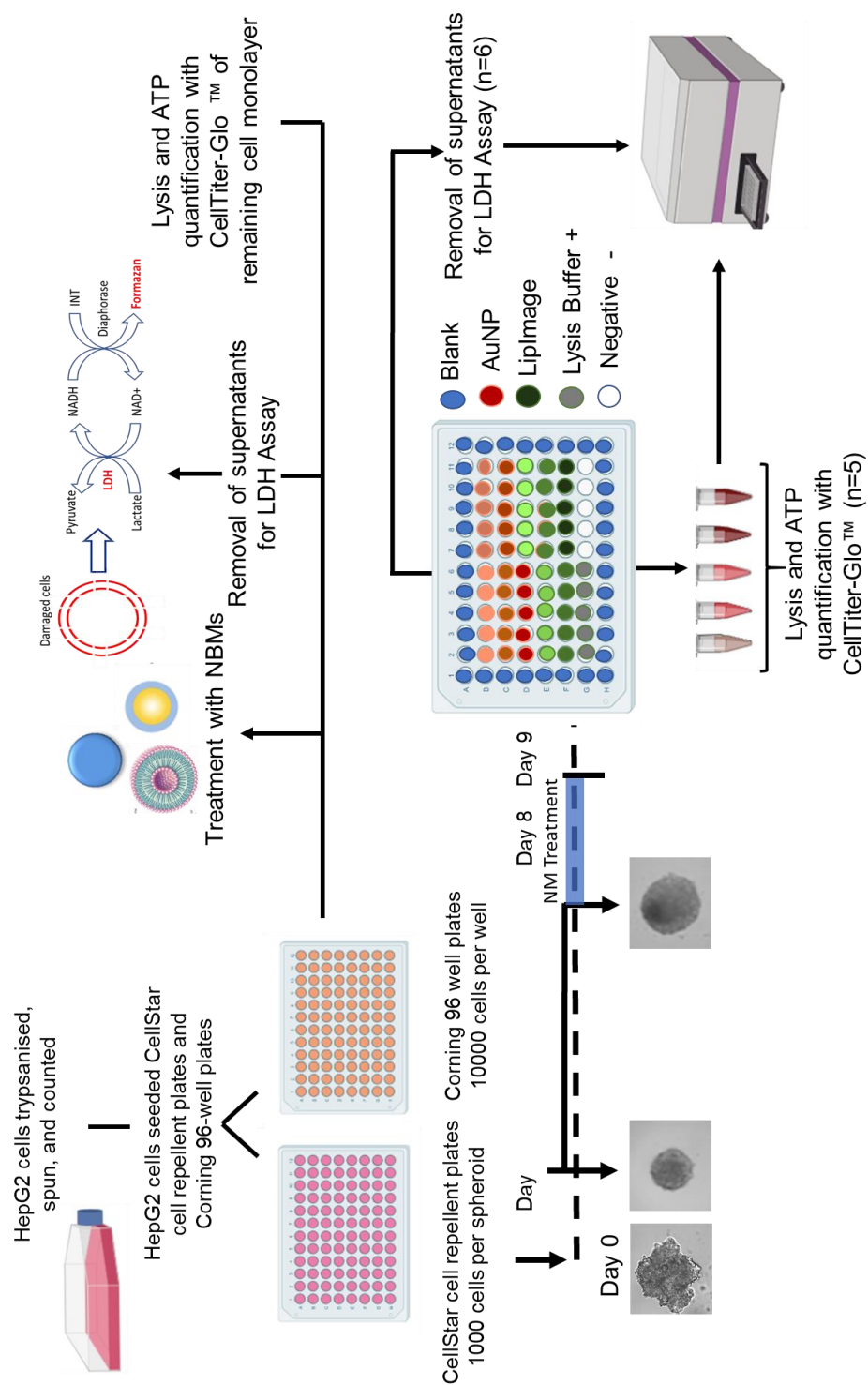
As the development of engineered nanoparticles is increasing at a rapid rate, reliable, robust and predictive *in vitro* methods of determining their potential toxicity are essential. For decades, studies have detailed these potential hazardous effects of various nanoparticles on mammalian cells cultures in 2D, however 2D cultures display many disadvantages including variation in cell morphology, function, lack of cell-cell contacts and cellular responses. For this reason, the development of more accurate and physiologically relevant models for mimicking *in vivo* conditions are vitally important. It is equally important to develop these models in an attempt to overcome the bottleneck currently seen with the translation of nanomaterial products to the clinic. For a nanomaterial-based drug product to make it through from pre-clinical assessment to use as a viable medicinal product, long optimization and testing procedures must be undertaken. Currently, two-thirds of all drugs that enter Phase II clinical trials, and one-third of drugs that enter Phase III fail to translate to in-human trials [504]. Similarly, the poor clinical translation of oncological nanomedicine products is one of the greatest challenges faced in modern nanomedicine. In contrast to other targeted anticancer drugs, e.g. vemurafenib, imatinib, multiple nanomedicine products have been approved in recent years which have proven safer and more effective than conventional cancer therapies. Despite this, the high turnover rate of more than 90% of new drug candidates entering clinical trials is of great concern. A lack of efficacy in humans accounts for approximately 60% of these failures, despite strong indications of robust therapeutic effects in pre-clinical assessment. It is safe to say that the translation rate of nanomedicine products into viable anticancer drugs is worryingly low. As previously mentioned, the limitations of pre-clinical assessment methods currently in use in the drug discovery pipeline may be at fault, contributing to the growing failure and poor translation of these drugs. Not only are traditional 2D cultures highly reductionist, failing to accurately recapitulate the *in vivo* environment, but doubts have also been raised as to the applicability of animal models in pre-clinical assessment, with many studies published in recent years detailing their inadequacies in accurately assessing efficacy of new drug candidates. For these reasons it is vitally important to integrate new preclinical assessment methods into the drug development pipeline.

Chapter 4 investigates whether 3D hepatic spheroids can have a higher predictive value than their 2D counterparts when screening NBM liver toxicity. This builds on the experimental evidence presented in Chapters 2 and 3 for the suitability of 3D *in vitro* hepatic spheroids to be adopted for biological relevance assessment for liver toxicity. In this chapter, 3D hepatic spheroids were exposed to varying concentrations of the chosen NBMs, namely an AuNP, LipImage and PACA, as detailed previously in Chapter 3. Changes in monoculture and multicellular spheroid viability (CellTiter-Glo[®] ATP quantification), cytotoxicity (LDH cytotoxicity assay) and genotoxicity (alkaline comet assay and γ H2AX staining) were assessed. NBMs were screened for their cytotoxic effects after continuous exposure at 24, 48 and 72 h; whereas, genotoxicity was assessed following 30 min, 3 h and 24 h exposure. Comparison was drawn to the changes detected in 2D hepatic monolayers for the same NBM concentrations, time-points, and experimental endpoints. By undertaking this work, it was possible to establish a validated safety workflow for the screening of lead NBMs which could be used for medical technology applications. Undertaken due to the overarching need for the development of robust and industrially acceptable methodologies for assessing the acute and chronic toxicity of NBMs, this work was completed within a large European Horizon 2020 project, REFINE (grant agreement ID: 761104), in conjunction with various other partners. This rationale behind this chapter was to establish protocols and assays which could be successfully applied to the assessment of NBM hepatotoxicity specifically in spheroid cultures, and in the case of the alkaline comet assay revising existing OECD guidelines [396]. To the best of my knowledge, this work is the first time these specific NBMs have been assessed in hepatic spheroid cultures, with regard to both cytotoxicity and genotoxicity.

4.2 Materials and Methods

The experimental workflows adopted for the work described within this chapter are illustrated in Figures 4.1 and 4.2.

All materials related to this chapter were purchased from Sigma Aldrich, Ireland, unless otherwise stated.



4.2.1 Cell Culture

HepG2 and LX-2 cells were maintained as per information provided in Chapter 2, Section 2.2.1. 2D monolayers and 3D spheroids were formed as described in Chapter 2, Section 2.2.2 and 2.2.3 respectively. For the work specifically described in this Chapter, 3D spheroids were formed using CellStar® plates only (1000 cell starting density in 100 µl culture medium), with spheroids cultured for 8d before treatments with NBMs. 2D monolayers were grown in 96 well plates for viability (10000 cells seeded in 100 µl culture medium per well), cytotoxicity (10000 cells seeded in 100 µl culture medium per well in 96 well plates) and DNA damage (2.5×10^5 cells/well in 2.5 ml culture medium in 6 well plates). Cells were cultured for 24 h before treatments.

4.2.2 NBM exposure

As there is the need to assess the acute and chronic impact of exposed NBMs, considerable effort has been invested in developing technically sound and industrially acceptable methodologies for screening these materials. These tasks were taken within a large European Horizon 2020 project among several partners. The REFINE project is focused on developing a scientific regulatory framework for NBMs. Thus, the experimental assays carried out for assessing the hepatic toxicity induced by NBMs are relevant to the project. Briefly, for each experimental endpoint, i.e. viability, cytotoxicity and genotoxicity, 2D and 3D cultures were exposed to AuNP, LipImage or PACA, using three of the concentration, as identified and reported in Chapter 3 (see section 3.3.3, Table 3.1), and extended to two additional lower concentrations, all reported in Table 4.1.

Table 4.1. Nanoparticles chosen for this study and concentrations used.

NBMs	Concentration (µg/ml)
AuNP	1, 5, 10, 20 and 30
LipImage™815	10, 50, 100, 200 and 500
NR668 PEBCA (PACA)	1, 5, 10, 20 and 30

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 or slides 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 timepoints, 24, 48 and 72 h. For comet assay, 2.5 ml of each NBM concentration diluted in culture medium was added to each well for three timepoints; 30 min, 3 h and 24 h (2D) and 24 h (3D). For γ H2ax staining, the highest concentration of each NBM was diluted in culture medium and 100 μ l was added to each well for 24 h.

4.2.3 ATP Quantification using the CellTiter-Glo™ Viability Assay

Cultures were washed with pre-warmed PBS and ATP content quantified using a luminescent cell viability assay (CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, MyBio Ltd, Ireland), performed as detailed in Chapter 2, Section 2.3.9.1. 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. Included in the experimental design were untreated controls (no NBMs, culture medium only), a 125 μ M Valinomycin positive control and a 10x lysis buffer positive control.

5.2.4 Lactate dehydrogenase (LDH) cytotoxicity assay

Supernatants were harvested from 2D cultures/3D monoculture and multicellular spheroids exposed to NBMs at three different timepoints, 24, 48 and 72 h. 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).

4.2.5 Alkaline Comet genotoxicity assay for assessing NBM-induced DNA damage

The alkaline comet assay was next to be developed for screening the nanocomponents of the selected NBMs. An experimental workflow was developed and is described in Figure 4.2. A standard protocol was designed to take into account all of the parameters impacting the assay so to harmonise the experimental findings. Cell cultures were exposed to NBMs for 30 min, 3 h and 24 h to trigger responses. Sublethal concentrations of each particle were chosen as Non-Adverse Observable Effect Levels (NOAEL) to show that NBMs can induce genetic damage even at non-cytotoxic doses. Six doses were determined from previous cytotoxicity assays as reported. Genotoxic positive control, 15 mM EMS, 30 min at 37°C, and a nanomaterial positive control, 10 µg/ml titanium dioxide (TiO₂) for 24 h at 37°C were included in study design as acquired from existing literature. A TiO₂ particle was chosen as a genotoxic positive control as they have widely been identified to impact DNA [397-399]. The alkaline comet assay for testing NBM genotoxicity was revised from the OECD Genetic Toxicology Test Guidelines document, as part of the revision discussion process [396] and is described in detail in the EMPA protocol [400]. Consequently, extensive discussion on the assay protocol for NBM screening was taken with EMPA and other European partners as part of the REFINE H2020 project activities on establishing new assay for assessing NBM-induced DNA damage (SOP available in Supplementary Section S.4.5).

In summary the new protocol is developed in the following steps. One day prior to cell harvest, frosted microscope slides were submerged in normal melting point agarose (1.5%) and excess wiped from underside. This was repeated 3 times. Slides were placed horizontally on bench to dry O/N. At the desired time point, cell culture supernatants were removed, and cells washed with pre-warmed PBS. TrypLE™ was used to harvest the hepatic cells (2D cultures: 0.2 ml; 3D spheroids: 50 µl/well). Cells/spheroids were incubated for 5 min before medium was added to neutralize TrypLE™ (0.3 ml for 2D and 75 µl for spheroids). The cell suspensions thus obtained were transferred to Eppendorf tubes and placed on ice. 160 µl low melting point (0.5%) agarose was mixed with 40 µl of cell suspensions in separate Eppendorf tubes by carefully pipetting up and down. 180 µl of the agarose-cell mixture was finally pipetted onto the agarose-coated slides, which were then cover slipped and incubated in the fridge for up to 2 h. Coverslips were removed, and slides placed in pre-cooled lysis solution for 1 h. Electrophoresis chamber was filled with electrophoresis buffer voltage fixed at power source to 24 V. Current was checked and adjusted to 300 mA by adapting buffer volume. After lysis, slides were taken from solution and drained carefully, before being placed submerged in electrophoresis chamber (ensuring labelled end of slide faces cathode).

DNA winding occurred for 20 min without current, before electrophoresis for an additional 20 min. After electrophoresis, slides were removed from chamber, drained and submerged in TRIS neutralization buffer for 5 min at RT. Slides were rinsed with double distilled water (ddH₂O) and then dehydrated by submerging them in absolute ethanol for 5 min at RT. Slides were then dried overnight at RT. 60 µl of a 20 µg/ml ethidium bromide (EtBr) solution was added per slide. Sides were sealed and imaged with a Nikon TE300 epifluorescent microscope and a 10X objective equipped with QCapturePro program and QImaging software, version 7.0.0.8. Exposure Acquisition values: 000.154.603 Ms, with Gain 3.870 and Offset -1374). In order to avoid re-scoring the same cells/comets more than once, a specific scoring pattern, seen in Figure 4.4, was implemented.

For use with hepatic spheroids, the same protocol was following, with amendments made to the dissociation protocol. Instead of using TrypLE™ to harvest cells, spheroids were incubated in 50 µl of Trypsin-EDTA for 10 min at 37°C, before mechanical dissociation using a 100 µl pipette tip.

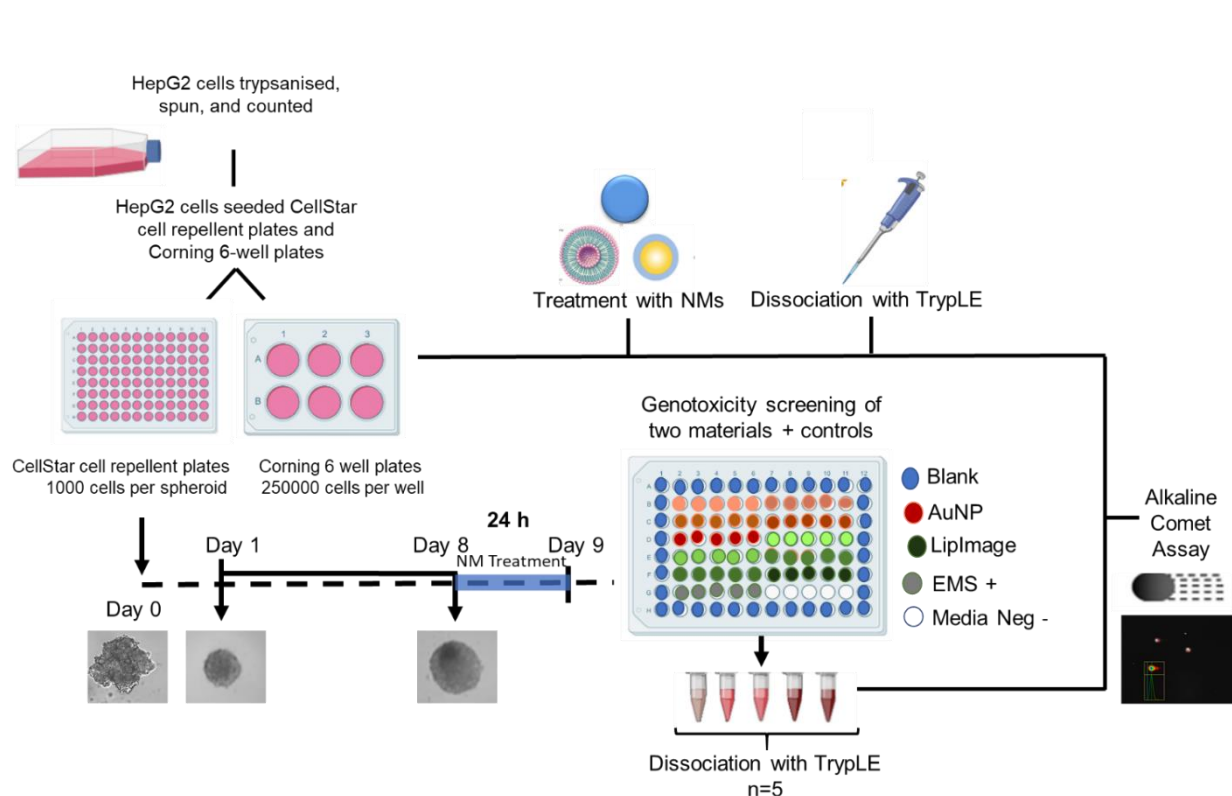


Figure 4.2. Schematic illustrating the experimental workflow developed and adopted for assessing the genotoxicity of NBMs in 2D and 3D cell models.

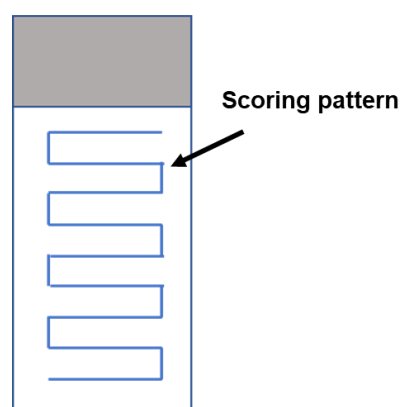


Figure 4.3. Scoring pattern adopted for the alkaline comet assay.

This specific scoring pattern is implemented to avoid re-scoring the same cells/comets and improving the reliability of the data obtained.

4.2.6 γ H2AX staining for monitoring NBM-induced DNA damage

The amount of DNA strand breaks induced by exposure to NBMs in both 2D monolayers and 3D spheroids was further analyzed qualitatively using γ H2AX staining and imaging. For 2D monolayers, cells were exposed to (i) 30 μ g/ml AuNP, (ii) 500 μ g/ml LipImage, and (iii) 30 μ g/ml PACA. An untreated control, two genotoxic positive controls and an NBM genotoxic positive control were included in study design. After 24 h exposure, cells were washed with pre-warmed PBS. Cells were then fixed with 3.7 % formaldehyde in PBS for 1 h at RT, before being permeabilized with 0.1 % Triton X-100 for 1 h at RT, and blocked with 5 % BSA in PBS for 30 minutes at RT. Cells were washed with PBS twice, before being stained with mouse anti-human phospho-histone H2AX (Ser139) primary antibody (Thermo Fisher Scientific, Ireland), diluted 1:200 in 5 % BSA in PBS, and incubated with the cells for 1 hour at RT. Cells were washed again with PBS, before staining with goat anti-mouse IgG (H+L) Cross-Adsorbed Secondary Antibody labelled with Alexa Fluor 532 (1:500) goat anti-mouse (Thermo Fisher Scientific, Ireland) and Hoechst (nuclei staining - 1:1000) (Thermo Fisher Scientific, Ireland) were both diluted 1:1000 in PBS. Cells incubated with secondary antibody at RT for 1 h, before being washed and mounted using SlowFade™ Gold Antifade Mountant (Thermo Fisher, Ireland). The same protocol was applied for 3D monoculture and multicellular spheroids, which were cultured for 8 d before being exposed to NBMs for 24 h. Imaging was undertaken using an epifluorescent microscope (Nikon TE300) equipped with a 100X objective, QCapture Software (QImaging) for 2D

monolayers; whereas LSCM and a 100x objective was used to image 3D spheroids (under the guidance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, using a Leica SP8 confocal microscope). Image processing for spheroids was undertaken using LASX software (Leica).

4.2.7 Statistical analysis

Data are representative of a minimum of at least three independent experiments (n=3) and expressed as mean $\pm$ SEM. All graphs and statistical analysis were obtained by GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA). Statistical test used is indicated in the figure captions.

4.3 Results

The results presented in this chapter focus on addressing the development and assessment of *in vitro* safety screening methodologies, for NBMs with medical applications.

4.3.1 Cell viability assessment of NBMs: comparison of exposure to 2D monolayers and 3D monoculture and multicellular spheroids

In order to determine if any significant differences were observed between both 2D and 3D cell models following treatment with the chosen panel of NBMs, cell/spheroid viability was assessed using the CellTiter-Glo™ ATP quantification assay.

4.3.1.1 ATP Quantification using the CellTiter-Glo® Viability Assay following treatment with 20 nm AuNP

Following NBMs treatment and removal of supernatants for LDH assay (Section 4.3.2), ATP content of the 2D monolayers and 3D monoculture and multicellular spheroids was quantified using the CellTiter-Glo® Luminescent Cell Viability Assay. Here, 40µl of CellTiter-Glo® reagent was added to washed wells for a 30 min incubation, followed by reading luminescence at 580nm. This assay was used to determine the presence of viable cells after NBM exposure and could be determined by presence of ATP, indicative of metabolically active cells. No significant cell death was observed in HepG2 monolayers following 24, 48 and 72h incubations, with ATP production comparable to that of the

untreated control. At the highest concentration, 30 µg/ml, % viability in HepG2 monolayers was $89.50\% \pm 4.15$, $91.17\% \pm 3.63$ and $86.58\% \pm 2.73$ after 24, 48 and 72h respectively, with any reduction in viability occurring in a dose and time dependent manner (Figure 4.4). Statistical analysis (two-way ANOVA with post-hoc Tukey test) revealed there was no significant drop in % viability at any of the chosen AuNP concentration and time point. When compared to HepG2 monolayers, HepG2 monoculture spheroids exhibited slightly greater % viability of $96.62\% \pm 3.92$, $87.94\% \pm 3.86$ and $89.99\% \pm 4.00$ after 24, 48 and 72h respectively following treatment with the highest concentration, 30 µg/ml. Again, statistical analysis revealed there was no significant drop in % viability at any of the chosen AuNP concentration and time point for HepG2 spheroids. Whilst QC is still being undertaken, it is suggested that the results for 2D monocultures were also comparable to *in vitro* cytotoxicity data undertaken by REFINE project collaborators using the same particle, assay and cell type (data to be presented at a later date). After treating HepG2/LX-2 multicellular spheroids with AuNP, % viability was $97.73\% \pm 2.89$, $87.17\% \pm 6.67$ and $86.95\% \pm 2.35$ after 24, 48 and 72h respectively, with no significant differences found when monoculture spheroids were compared to multicellular spheroids. The incorporation of LX-2 cells into the model therefore had no impact on cell viability following treatment with AuNPs.

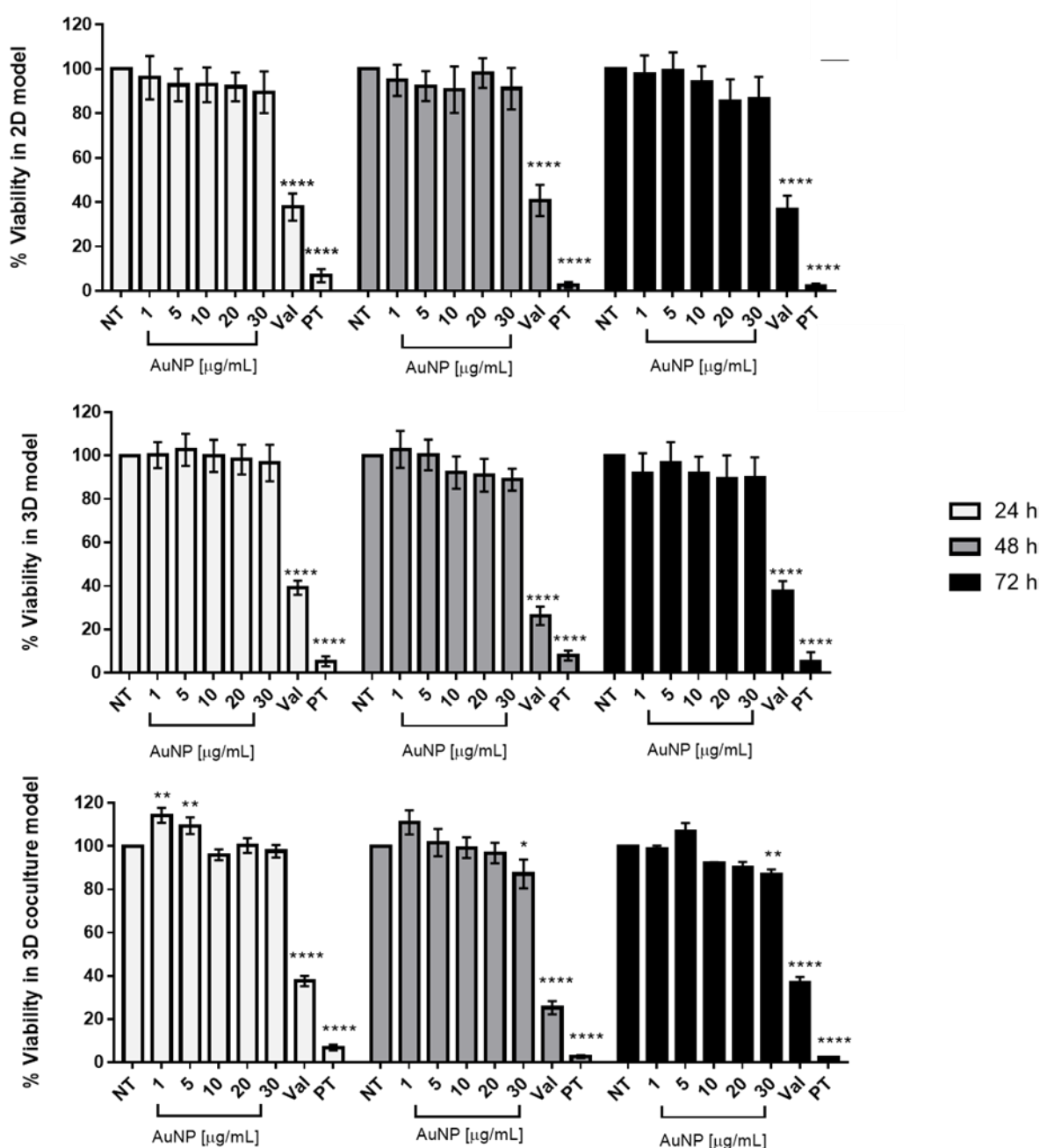


Figure 4.4. Evaluation of cell viability in AuNP treated 2D hepatic monocultures and 3D monocellular and multicellular (co-culture) spheroids using the CellTiter-Glo™ ATP quantification assay.

(A) 2D HepG2 monocultures, (B) 3D HepG2 spheroids, and (C) 3D HepG2/LX-2 multicellular spheroids following exposure to increasing concentrations (1 – 30 μg/ml) of AuNP after 24, 48 and 72h. Data is expressed as percentage of viable cells and representative of a minimum of three independent experiments (n=3) and expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Statistical analysis by Two-

way ANOVA, with post hoc Tukey test. All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

Table 4.2. *p* values for individual *t* tests carried out for different culture conditions at each NBM concentration.

2D = 2D monoculture, 3D mono = 3D monoculture spheroids, 3D multi = 3D multicellular spheroids. Post hoc analysis carried out using Bonferroni-Dunn method, ***p*<0.01. All graphs and statistical analysis were undertaken in GraphPad Prism 7.

		NT	1	5	10	20	30	Val	PT
24 h	2D v. 3D mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	0.004 **	0.009 **	ns	ns	ns	ns	ns
48 h	3D mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
72 h	2D v. 3D mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns

4.3.1.2 ATP Quantification using the CellTiter-Glo® Viability Assay following treatment with LipImage™815.

Viability of monolayers and spheroids also remained constant following treatment with LipImage, with significant drops in ATP production only observed at the greater concentrations, i.e., 100, 200 and 500 µg/ml, for each time point respectively (Figure 4.5). Lowest % viability for 2D monolayers were 88.45% ± 2.92, 87.58% ± 2.39, and 82.68% ± 2.93 after 24, 48 and 72h exposures, for 500 µg/ml treatments. When compared to HepG2 monolayers, HepG2 monoculture spheroids exhibited greater % viability, with 100.91% ± 5.21, 90.62% ± 7.5 and 91.47% ± 9.53 after 24, 48 and 72h respectively. Statistical analysis revealed there was no significant drop in % viability at any of the chosen LipImage concentrations and time point for HepG2 spheroids. The results obtained for 2D monolayer cultures were comparable to *in vitro* cytotoxicity data undertaken by collaborators in the REFINE project through an interlaboratory comparison study, using the same particle, assay and cell type (QC ongoing, data not shown and to be presented at a later date). Following

treatment of HepG2/LX-2 multicellular spheroids with LipImage, % viability once again remained similar to monoculture spheroids, with each concentration having no significant impact on spheroid viability. % viability following 500 µg/ml treatment was $94.24\% \pm 7.82$, $95.01\% \pm 1.46$, and $90.59\% \pm 1.52$ after 24, 48 and 72h respectively. No significant differences were found between any of the culture types, at any of the three time points.

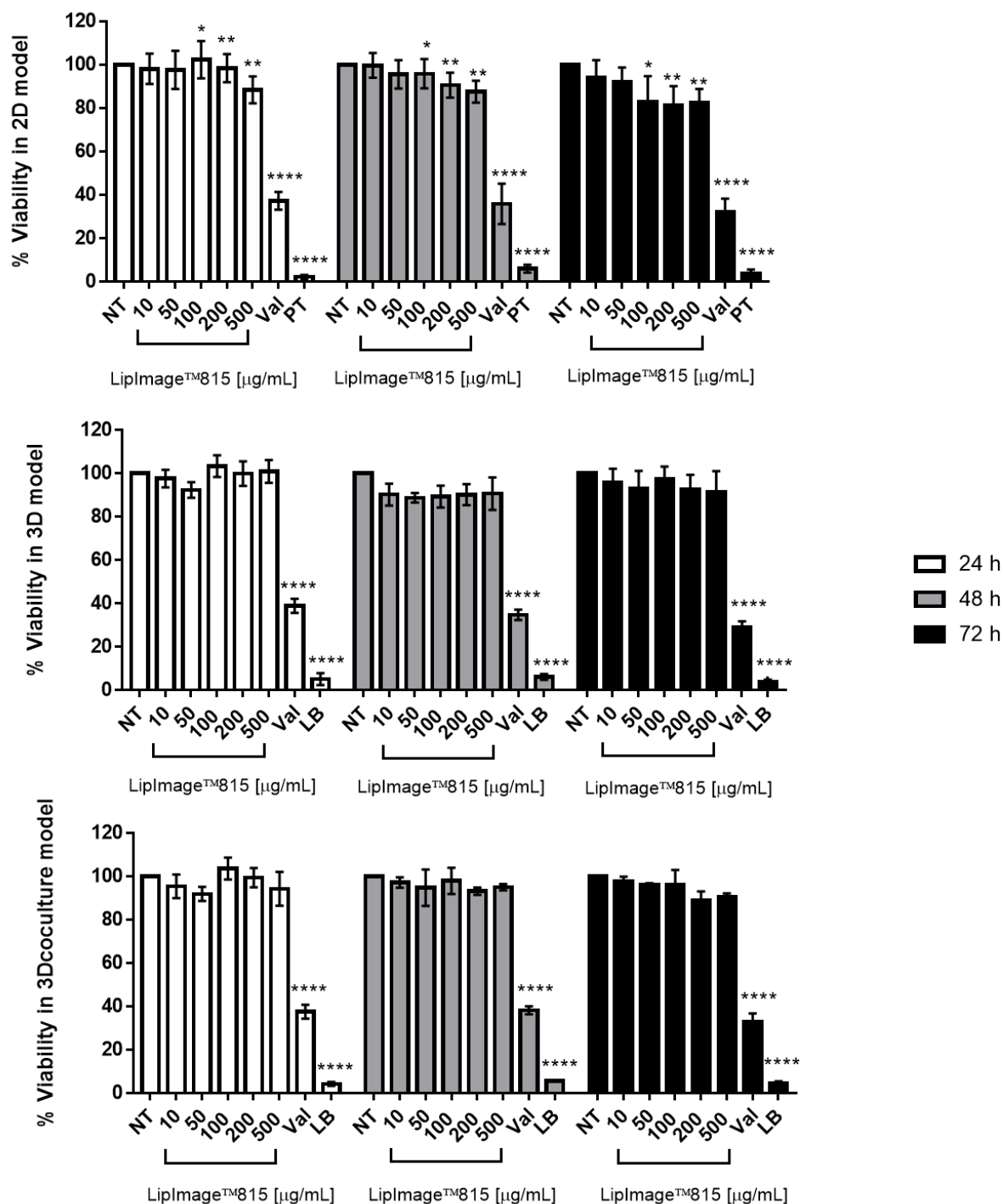


Figure 4.5. Evaluation of cell viability in LipImage treated hepatic 2D monocultures and 3D monocellular and multicellular (co-culture) spheroids using the CellTiter-Glo™ ATP quantification assay.

(A) 2D HepG2 monocultures, (B) 3D HepG2 spheroids, and (C) 3D HepG2/LX-2 multicellular (co-culture) spheroids following exposure to increasing concentrations (10 – 500 µg/ml) of LipImage after 24, 48 and 72h. Data is expressed as percentage of viable cells

and representative of a minimum of three independent experiments ($n=3$) and expressed as mean $\pm$ SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. Statistical analysis by Two-way ANOVA, with post hoc Tukey test. All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

Table 4.3. *p* values for individual *t* tests carried out for different culture conditions at each NBM concentration

Post hoc analysis carried out using Bonferroni-Dunn method. All graphs and statistical analysis were undertaken in GraphPad Prism 7.

		NT	1	5	10	20	30	Val	PT
24 h	2D v. 3D	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. Coculture	ns	ns	ns	ns	ns	ns	ns	ns
	3D v. Coculture	ns	ns	ns	ns	ns	ns	ns	ns
48 h	2D v. 3D	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. Coculture	ns	ns	ns	ns	ns	ns	ns	ns
	3D v. Coculture	ns	ns	ns	ns	ns	ns	ns	ns
72 h	2D v. 3D	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. Coculture	ns	ns	ns	ns	ns	ns	ns	ns
	3D v. Coculture	ns	ns	ns	ns	ns	ns	ns	ns

4.3.1.3 ATP Quantification using the CellTiter-Glo® Viability Assay following treatment with PACA

The final NBM screened was PACA, which exhibited the greatest drop in cell viability. This was most significant in the 2D cultures, where % viability following treatment with 30 µg/ml was $79.76\% \pm 1.63$, $85.23\% \pm 5.47$, and $73.55\% \pm 4.06$ following 24, 48 and 72h treatments respectively (Figure 4.6). When 2D cultures are compared to the spheroid models, the monoculture spheroids were shown to exhibit greater % viability, with $91.66\% \pm 5.91$, $84.918\% \pm 2.54$ and $81.47\% \pm 4.81$ after 24, 48 and 72h treatments of 30 µg/ml.

The HepG2/LX-2 multicellular model exhibited greater viability than both the 2D monoculture and monoculture spheroid model, however this was only significant between 2D and co-culture after 30 µg/ml treatment at 24h ($***p<0.001$) and after 20 µg/ml treatment after 72 h ($**p<0.01$). % viability following treatment with the largest concentration (30 µg/ml) was $95.05\% \pm 1.39$, $90.56\% \pm 1.57$ and $87.68\% \pm 8.17$ after 24, 48 and 72h respectively. Once again, it is suggested that the results obtained for 2D monolayer cultures are comparable to *in vitro* cytotoxicity data undertaken by collaborators in the REFINE project through an interlaboratory comparison using PACA (QC ongoing, data to be presented at a later date).

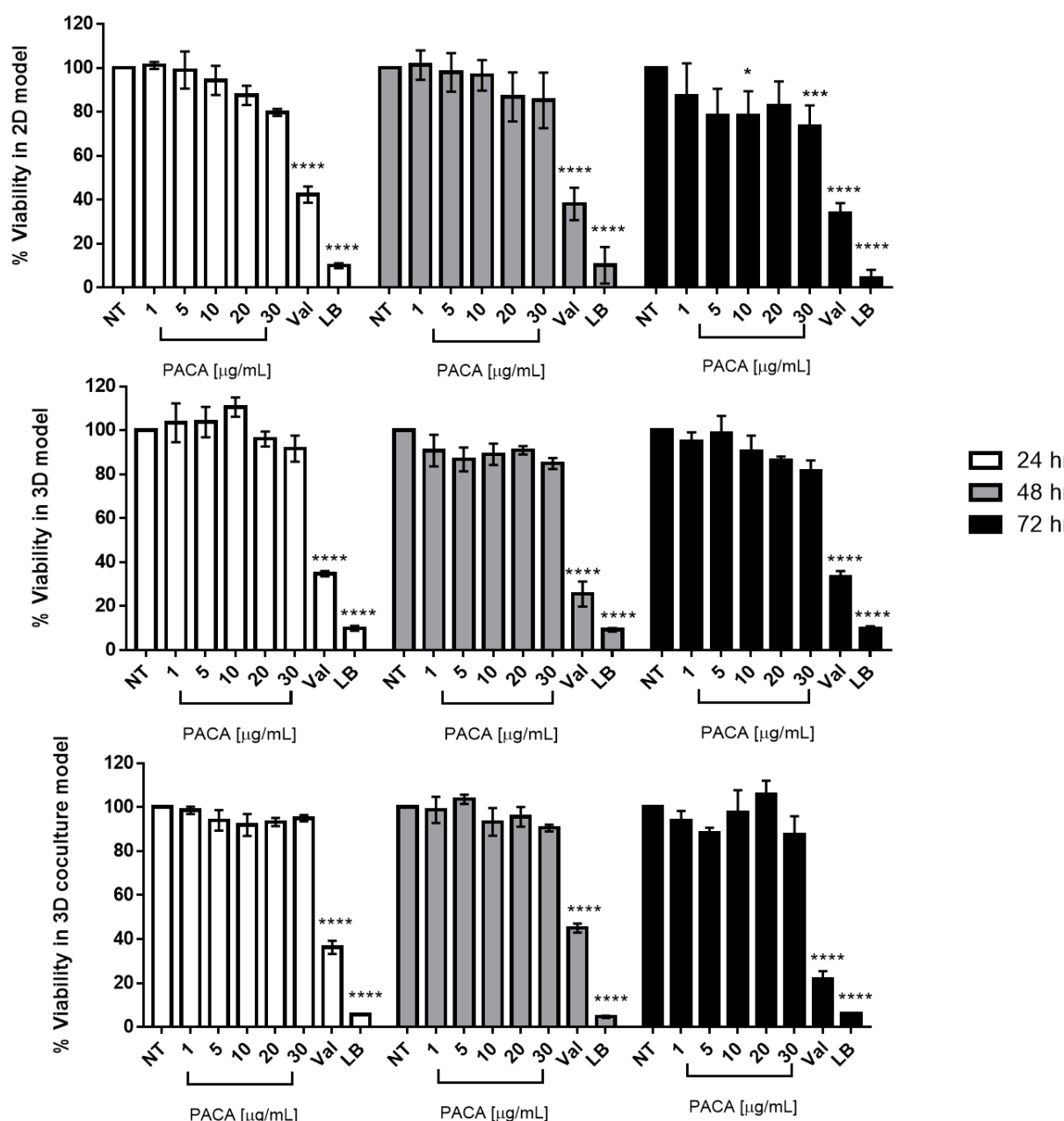


Figure 4.6. Evaluation of cell viability in PACA (NR668 PEBCA) treated hepatic 2D monocultures and 3D monocellular and multicellular (co-culture) spheroids using the CellTiter-Glo™ ATP quantification assay.

(A) 2D HepG2 monocultures, (B) 3D HepG2 spheroids, and (C) 3D HepG2/LX-2 multicellular (co-culture) spheroids following exposure to increasing concentrations (1 – 30 µg/ml) of PACA after 24, 48 and 72h. Data is expressed as percentage of viable cells and representative of a minimum of three independent experiments (n=3) and expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Statistical analysis by Two-way ANOVA, with post hoc Tukey test. All graphs and statistical analysis were

undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

Table 4.4. *p* values for individual *t* tests carried out for different culture conditions at each NBM concentration.

Post hoc analysis carried out using Bonferroni-Dunn method, ***p* < 0.01 and ****p* < 0.001.

All graphs and statistical analysis were undertaken in GraphPad Prism 7.

		NT	1	5	10	20	30	Val	PT
24 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	***0.00067	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
48 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
72 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	**0.008	ns	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns

4.3.2 Membrane damage induced by NBMs exposure in 2D monolayers and 3D monoculture and multicellular spheroids of HepG2 cells

To assess NBM-induced cytotoxicity, the lactate dehydrogenase (LDH) assay was used. This assay was chosen as analysis was undertaken on collected supernatants, therefore avoiding the issues seen with conventional cytotoxicity assays and NBM interference, such as MTT or WST-8. LDH is a cytosolic enzyme released in response to plasma membrane damage. Extracellular LDH in culture medium is quantified by a coupled enzymatic reaction where LDH catalyses the conversion of lactate to pyruvate, via NAD⁺ reduction to NADH. NADH is then used to reduce a tetrazolium salt into a red formazan product, which can be measured fluorescently at 490nm (Figure 4.7). The level of formazan formed is directly proportional to LDH released into the supernatants and is therefore indicative of cytotoxicity. Untreated cultures were included in the experimental design and used as negative controls (NT). Exposure of cells to LDH lysis buffer for 45 min was used as a positive control (PT).

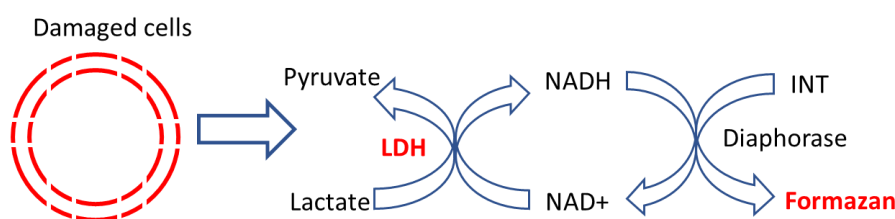


Figure 4.7. Schematic representing LDH cytotoxicity assay mechanism.

LDH, a soluble cytosolic enzyme, is released into culture medium from damaged cells upon cell death due to plasma membrane damage, with increased LDH activity in supernatants proportional to number of damaged, lysed cell. LDH catalyses the reduction of NAD⁺ to NADH, in the presence of L-lactate, with the formation of NADH measured in a coupled reaction whereby the tetrazolium salt, INT, is reduced to a red formazan product. The amount of the strongly coloured and soluble formazan is then measured at 490 nm spectrophotometrically. How does LDH Cytotox relate to hepatocyte modulation

4.3.2.1 Lactate dehydrogenase (LDH) cytotoxicity assay for AuNP-induced cytotoxicity

Cytotoxicity was assessed using cell culture supernatants collected after 24, 48 and 72 h post-NBMs exposure, from 2D monolayer and 3D monoculture and multicellular spheroids, with results normalised to maximum LDH release (lysis buffer wells). For the AuNP it could be determined that cytotoxicity occurs in a time and concentration dependent manner, with an increase in LDH release only measured as treatment time progresses (Figure 4.8). In 2D monolayers, only small amounts of LDH were released, with no significant cytotoxicity detected for the 20nm AuNPs at any of the concentrations or time points. The highest cytotoxicity is seen at 20 µg/ml from the 72h exposure, with $6.59\% \pm 2.27$ cytotoxicity level in HepG2 monolayers. This release occurred in a dose-dependent manner and is in line with literature which suggests that smaller sized AuNPs (<20 nm) in general induce less membrane damage and LDH leakage than your larger counterparts but do induce higher LDH secretion at higher concentrations. Preliminary analysis suggests that these results were also comparable to *in vitro* cytotoxicity data undertaken by collaborators in the REFINE project, using the same particle, assay and cell type (QC ongoing, data to be presented at a later date).

In order to determine if altered cytotoxicity and enhanced sensitivity could be observed in 3D spheroids in comparison to 2D HepG2 monolayers, 3D monoculture spheroids and HepG2/LX-2 multicellular spheroids were treated with the same particles and concentrations as for 2D monolayers, supernatants removed and LDH assay undertaken. In monoculture HepG2 spheroids no cytotoxicity was observed (Figure 4.8) after 24h exposure, other than the 10 µg/ml concentration ($3.83\% \pm 1.24$), and the highest concentration (30 µg/ml) where $2.12\% \pm 0.45$ cytotoxicity level was measured. In 3D monoculture spheroids, no cytotoxicity was noted until the highest concentration, 30 µg/ml, with % cytotoxicity $1.93\% \pm 0.52$. Greater cytotoxicity was found in HepG2/LX-2 multicellular spheroids, with $5.50\% \pm 15.19$ and $2.52\% \pm 7.20$ cytotoxicity observed following 10 and 30 µg/ml treatments respectively. No significant differences were seen between any of the culture types after 24h. Following 48 h exposure, a proportional increased in % cytotoxicity was detected in 2D cultures, with 30 µg/ml inducing the greatest cytotoxicity, i.e. $5.21\% \pm 1.05$. The same pattern was observed in 3D monoculture spheroids, with % cytotoxicity $4.18\% \pm 0.94$ following 30 µg/ml treatment. In multicellular spheroids, the greatest cytotoxicity was following 20 µg/ml treatment, i.e. $5\% \pm 1.74$ cytotoxicity, followed by the 30 µg/ml treatment, $4.02\% \pm 1.25$. The only significant difference between cultures treated with AuNP was between the

monoculture 3D spheroids and the coculture spheroids, where $*p < 0.05$ (t tests followed by post hoc Bonferroni-Dunn method) (Table 4.5). Following 72h incubation with AuNP, the greatest % cytotoxicity in 2D cultures was seen following 20 $\mu\text{g/ml}$ treatment ($6.59\% \pm 2.27$). Greatest % cytotoxicity in 3D monoculture spheroids was also from the 20 $\mu\text{g/ml}$ treatment, with $5.83\% \pm 1.92$ cytotoxicity observed. Less cytotoxicity was found in multicellular spheroids compared to the other cultures, with $4.71\% \pm 0.54$ and $4.57\% \pm 1.36$ recorded for the 20 and 30 $\mu\text{g/ml}$ treatments respectively. No significant differences were seen between any of the culture types after 72h. Preliminary analysis suggests that for 2D monolayer cultures, results obtained were comparable to LDH assay undertaken by collaborators in the REFINE project using the same particle, assay and cell type (QC ongoing, data to be presented at a later date).

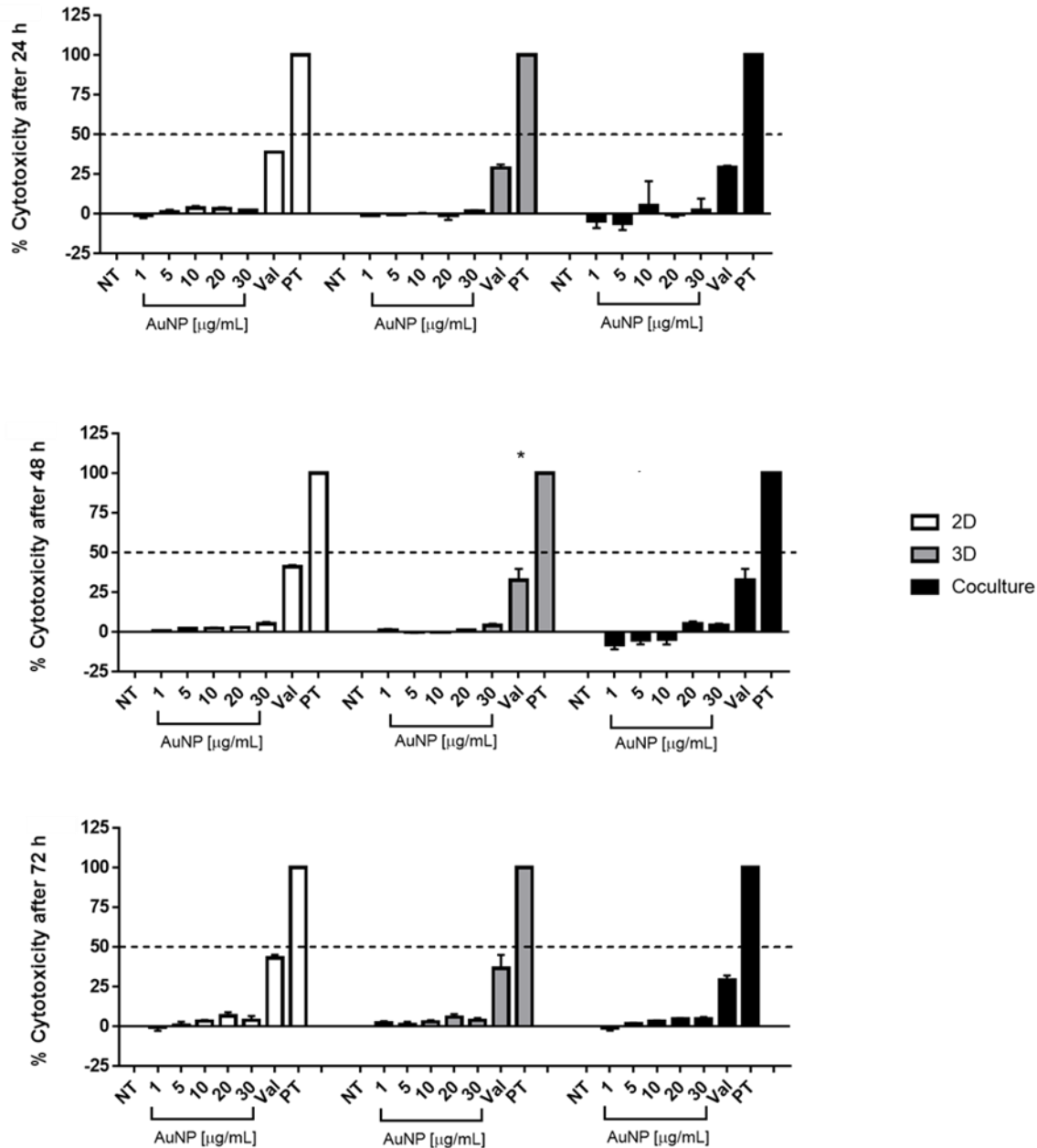


Figure 4.8. Cytotoxicity induced in HepG2 2D monolayers and 3D monocellular and multicellular (co-culture) spheroids following (A) 24, (B) 48 and (C) 72h exposures to AuNP.

LDH assay calculated to show % cytotoxicity induced by AuNP following 24, 48 and 72h incubations in each of the culture methods. Data is expressed as percentage of viable cells and representative of a minimum of three independent experiments ($n=3$) and expressed as mean $\pm$ SEM. * $p = 0.05$ for 3D monoculture v 3D multicellular models, for 1 $\mu\text{g/mL}$ 48 h

(*).All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

Table 4.4. *p* values for individual *t* tests carried out for different culture conditions at each NBM concentration.

Post hoc analysis carried out using Bonferroni-Dunn method, **p* < 0.05.

		NT	1	5	10	20	30	Val	PT
24 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
48 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D Mono v. 3D Multi	ns	0.0399*	ns	ns	ns	ns	ns	ns
72 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns

4.3.2.2 Lactate dehydrogenase (LDH) cytotoxicity assay for LipImage™815-induced cytotoxicity

After 24 h treatment with LipImage, no cytotoxicity was observed until 500 µg/ml treatment in both 2D cultures and 3D multicellular spheroids (Figure 4.9); 1.69% ± 0.87 and 2.53% ± 6.51 respectively. No significant differences were found after 24h between any of the different culture types (Table 4.6). Following 48h incubation, no cytotoxicity was observed for 2D cultures until the largest concentration, 500 µg/ml, where % cytotoxicity was 1.69% ± 0.87. A similar pattern was observed for 3D monoculture spheroids, where largest % cytotoxicity was observed following 500 µg/ml treatment; 1.9% ± 0.52. QC is ongoing, however preliminary analysis suggests that these results are comparable to *in vitro* cytotoxicity data undertaken by collaborators at SINTEF through an interlaboratory comparison, using the same particle, assay and cell type (data to be presented at a later date). The only significant difference noted for all LipImage experiments was observed after 48h between 2D culture and 3D multicellular spheroids, **p*<0.05 (t tests followed by post hoc Bonferroni-Dunn method). After 72h, a dose-dependent increase in LDH secretion was

recorded, with $7.38\% \pm 3.82$ and $10.83\% \pm 2.54$ cytotoxicity observed for 200 and 500 $\mu\text{g/ml}$ concentrations. After 72h incubation, a dose-dependent increase in % cytotoxicity was observed for 2D cultures, with $0.86\% \pm 6.98$, $7.38\% \pm 3.82$ and $10.83\% \pm 2.54$ observed for 100, 200 and 500 $\mu\text{g/ml}$ concentrations respectively. No cytotoxicity was detected until the 500 $\mu\text{g/ml}$ concentration in 3D monoculture spheroids, where % cytotoxicity was $1.78\% \pm 1.92$. In HepG2/LX-2 multicellular spheroids, all concentrations induced mild cytotoxicity, ranging from $1.99\% \pm 1.14$ at 10 $\mu\text{g/ml}$ to $6.75\% \pm 0.34$ at 500 $\mu\text{g/ml}$. No significant differences were recorded after 72h between any of the different culture types. QC is ongoing, however preliminary analysis suggests that these results are comparable to *in vitro* cytotoxicity data undertaken through an interlaboratory comparison with collaborators at SINTEF, using the same particle, assay and cell type (data to be presented at a later date).

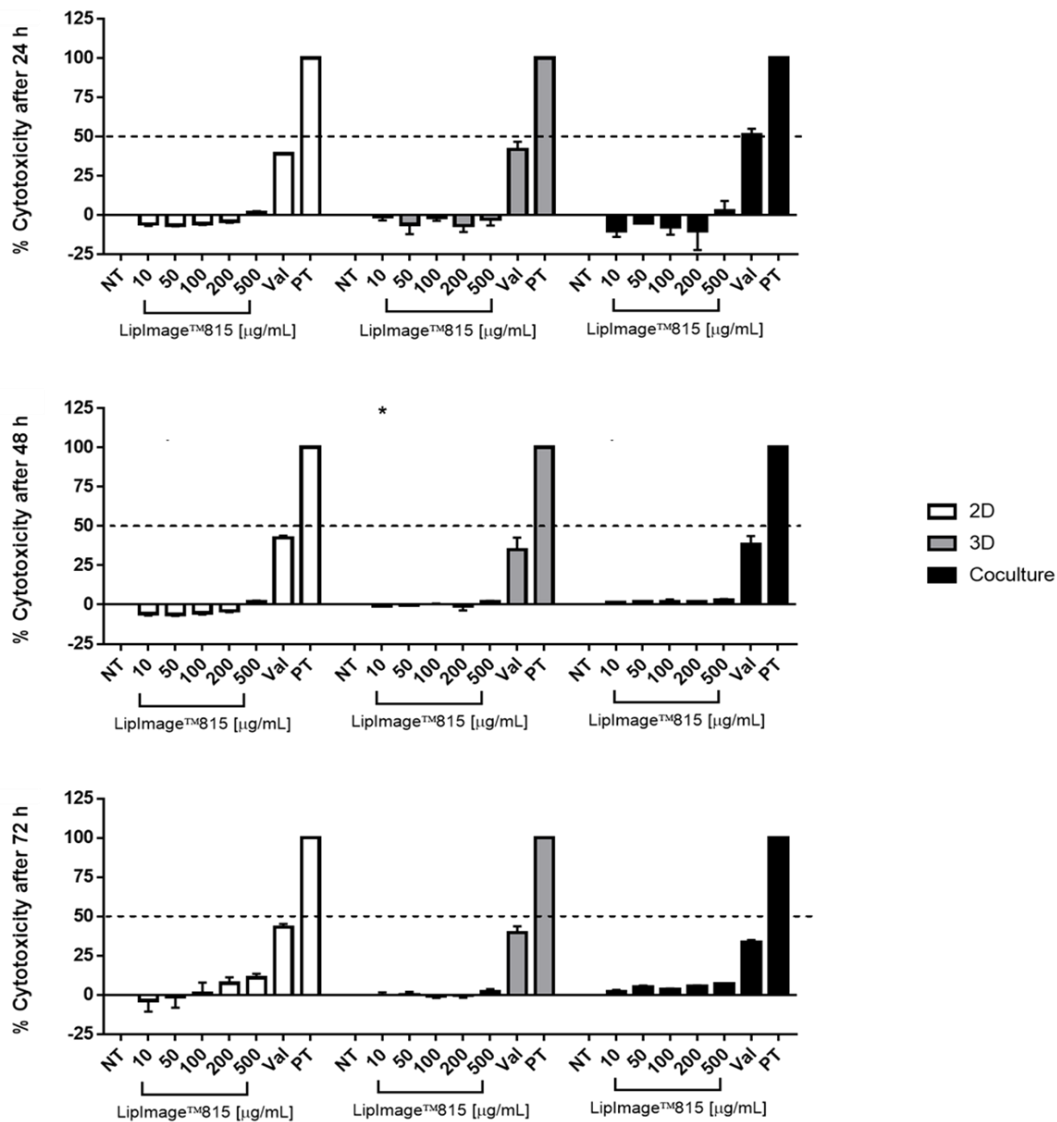


Figure 4.9. Cytotoxicity induced in HepG2 2D monolayers and 3D monocellular and multicellular (co-culture) spheroids following (A) 24, (B) 48 and (C) 72h exposures to LipImage.

LDH assay calculated to show % cytotoxicity induced by LipImage following 24, 48 and 72h incubations in each of the culture methods. Data is expressed as percentage of viable cells and representative of a minimum of three independent experiments (n=3) and expressed as mean $\pm$ SEM. * $p = 0.05$ for 2D monolayer v 3D monoculture models, for 50 $\mu\text{g/mL}$ 48 h (*) Statistical analysis by Two-way ANOVA and Bonferroni-Dunn method. All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

Table 4.5. p values for individual t tests carried out for different culture conditions at each NBM concentration.

Post hoc analysis carried out using Bonferroni-Dunn method, * $p < 0.05$.

		NT	10	50	100	200	500	Val	PT
24 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
48 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	*0.0472	ns	ns	ns	ns	ns
	3D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
72 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns

4.3.2.3 Lactate dehydrogenase (LDH) cytotoxicity assay for PACA-induced cytotoxicity

The final particle assessed was the NR668 PEBCA (PACA). , After 24h treatment with PACA, % cytotoxicity was observed after 10, 20 and 30 $\mu\text{g/mL}$ exposures in 2D culture with % cytotoxicity $4.51\% \pm 0.72$, $7.51\% \pm 4.26$ and $6.81\% \pm 3.11$ respectively (Figure 4.10) In 3D cultures, less cytotoxicity was detected, with $2.57\% \pm 0.90$ and $2.35\% \pm 0.12$ observed

following 20 and 30 µg/ml exposures. No cytotoxicity was recorded when multicellular spheroids were treated with PACA, with the only significant difference between each culture observed between 2D culture and HepG2/LX-2 multicellular spheroids where $*p < 0.05$ at 20 µg/ml concentration. Following 48h incubation, a concentration dependent increase in cytotoxicity was observed in both 2D and 3D monocultures, with greatest % cytotoxicity after 30 µg/ml treatment ($7.10\% \pm 1.18$ for 2D and $3.49\% \pm 1.89$ for 3D). The greatest cytotoxicity seen in the multicellular model, with $4.37\% \pm 0.99$ and $3.33\% \pm 0.71$ observed for 20 and 30 µg/ml treatments. No significant differences were seen after 48h between any of the different culture types. The greatest significant differences were detected after 72h treatments. A dose dependent increase in cytotoxicity was observed in both 2D and 3D monocultures, with 20 and 30 µg/ml doses yielding $15.75\% \pm 4.31$ and $20.87\% \pm 3.43$ cytotoxicity in 2D cultures respectively. Cell death was markedly reduced in both 3D mono- and multicellular spheroids. In 3D spheroids, greatest cytotoxicity was noted after 30 µg/ml, $8.30\% \pm 2.65$, and in co-culture spheroids 10 µg/ml caused the greatest cell death ($5.26\% \pm 2.43$).

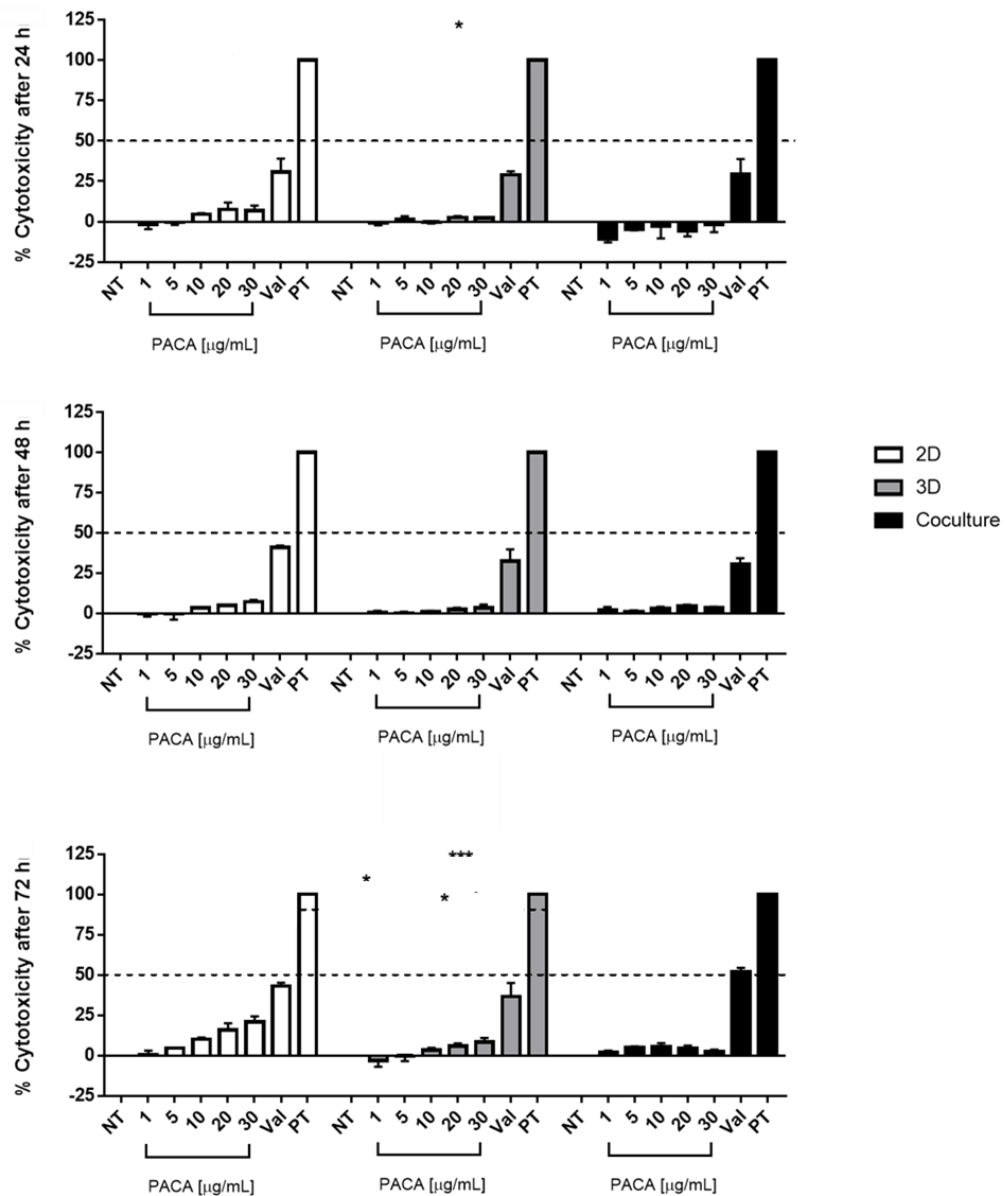


Figure 4.10. Cytotoxicity induced in HepG2 2D monolayers and 3D monocellular and multicellular spheroids following (A) 24, (B) 48 and (C) 72h exposures to PACA.

LDH assay calculated to show % cytotoxicity induced by PACA following 24, 48 and 72h incubations in each of the culture methods. Data is expressed as percentage of viable cells and representative of a minimum of three independent experiments ($n=3$) and expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, for 2D monolayer v 3D multicellular 20 $\mu\text{g/mL}$ 24 h (*), 2D monolayer v.3D multicellular 20 $\mu\text{g/mL}$ 72 h (*), 2D monolayer v. 3D monoculture 30 $\mu\text{g/mL}$ 72 h (*), and 2D monoculture v. 3D multicellular 30 $\mu\text{g/mL}$ 72 h (****). Statistical analysis by Two-way ANOVA and Bonferroni-Dunn method. All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

Table 4.6. *p* values for individual *t* tests carried out for different culture conditions at each NBM concentration.

*Post hoc analysis carried out using Bonferroni-Dunn method, **p* < 0.05, *****p* < 0.0001.*

		NT	1	5	10	20	30	Val	PT
24 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	*0.0243	ns	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
48 h	2D v. 3D Mono	ns	ns	ns	ns	ns	ns	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
	3D Mono v. 3D Multi	ns	ns	ns	ns	ns	ns	ns	ns
72 h	2D v. 3D	ns	ns	ns	ns	ns	*0.0102	ns	ns
	2D v. 3D Multi	ns	ns	ns	ns	*0.0220	****0.0002	ns	ns
	3D Mono v. 3D	ns	ns	ns	ns			ns	ns
	Multi					ns	ns		

4.3.3 Development of a SOP for evaluating the DNA damage in 3D hepatic spheroids exposed to NBMs via alkaline comet assay

Despite the pre-screening assessment for NBMs incorporating many key assays for testing NBM safety, e.g., cytotoxicity and immunotoxicity; it emerged genotoxicity is largely overlooked. To date no standardised and validated SOP for assessing NBM-induced DNA damage exists. This was identified as a regulatory gap and opportunity to be developed within the REFINE project. During my project, as part of the Horizon 2020 REFINE project and in consultation with the other partners involved, namely EMPA and IBE centre, I worked at developing an SOP for assessing DNA damage induced by NBMs using the alkaline comet assay. This SOP was validated by an internal round robin interlaboratory comparison study with three partners (TCD, SINTEF and IBE), including other partners in the REFINE project, and will be also be adopted from January 2021 in an external round robin study. More information on this interlaboratory comparison study can be found at <http://refine-nanomed.eu/external-inter-laboratory-comparison/>, and the full developed SOP can be found in Supplementary Section 4.5).

Briefly, the comet assay has achieved the status of a standard test, in the cascade of assays used to assess the safety of novel pharmaceuticals, nanomaterials and other chemicals [401]. Due to the adverse effects of DNA strand breaks to human health, assessing mutagenic potential of nanomaterials is a vital component of nano risk assessment. The SOP developed details an *in vitro* method for evaluating genotoxicity in the HepG2 cell line following exposure to NBM formulations. At present, all types of nanoparticle formulation are suitable for assessment using this protocol as long as there is no particle interference with nuclear DNA in its unwinding state, i.e., there should be no interfere with microscopic fluorescence readouts. Comet assay IV software is recommended as part in the SOP for assessing image readouts and scoring comets, as due to the automated nature of this software any subjectivity or bias is removed. This avoids issues often observed when using a less automated software such as ImageJ.

4.3.4 Assessing double strand breaks induced by NBMs in 3D spheroids and 2D monolayers of HepG2 cells using the alkaline comet assay and developed SOP

2D HepG2 monolayers and 3D monoculture and multicellular spheroids were exposed to the three NBMs (AuNP, LipImage and PACA), using the aforementioned SOP presented in full details in the Supplementary Section 4.5, to measure the their potential to induce DNA

strand breaks, i.e. genotoxicity, was assessed. Genotoxicity is a crucial endpoint in determining the overall toxicity of any NBM, and the alkaline comet assay has proved to be a sensitive and simple method of determining DNA damage. This is currently the most widely used method for genotoxicological assessment of nanomaterials due to its robustness, reliability, and versatility. 24, 48 and 72h exposures were undertaken, with multiple non-cytotoxic concentrations tested, ranging from 1 – 30 µg/ml for AuNP and PACA, and 5 – 500 µg/ml for LipImage. For each experiment a genotoxic positive control, 15 mM EMS, a nanoparticle control, 10 µg/ml TiO₂, and a negative control (cell culture medium alone) were also tested. Representative images of comet scoring in positive and negative controls using the CometIV lite software (Instem, United Kingdom), and examples of comets which should not be scored, i.e. ‘ghost’ comets or over confluent areas can be found in Supplementary Section Figures S4.5.

4.3.4.1 Assessing NBM-induced double strand breaks in 2D monocultures using the SOP developed for the alkaline comet assay

Compared to negative control levels ($1.92\% \pm 0.42$, $1.64\% \pm 0.65$ and $1.49\% \pm 0.36$ for 24, 48 and 72h respectively), the AuNP treatment caused no significant DNA breaks in monolayer HepG2 cells, as presented in Figure 4.11. No concentration caused DNA damage above the 10% QC criteria, with largest % of DNA damage noted following the 30 min incubation. Incubation of cells with LipImage also caused no notable DNA damage at concentrations ranging from 0 – 100 µg/ml but appears genotoxic at a low level at the higher concentrations, 200 and 500 µg/ml, following both 3 hr and 24 h incubation, as illustrated in Figure 4.12. However, the % tail values for these concentrations remain less than 10% DNA, which is in accordance with QC/QA criteria. LipImage also expresses genotoxicity in a dose-dependent manner. No notable DNA damage was indicated following treatments with PACA at any of the time points assessed, with all % tail values below the 10% tail value QC criteria. All positive controls (cells treated with 15 mM EMS or 10 µg/ml TiO₂) showed increased DNA strand breaks in all experiments, with % tail values as high as $48.98\% \pm 0.78$ for EMS and $5.54\% \pm 1.58$ for TiO₂ respectively. Following treatment with PACA (Figure 4.13), no concentration caused DNA damage above the 10% QC criteria, with largest % of DNA damage occurring after 30 min treatment with 5 µg/ml ($4.3\% \pm 2.21$). Whilst QC is ongoing, analysis suggests that the results obtained for 2D monolayer cultures treated with all NBMs were comparable to genotoxicity data obtained as part of the round robin study previously

detailed, undertaken as part of the REFINE project (data not shown, to be reproduced at a later date).

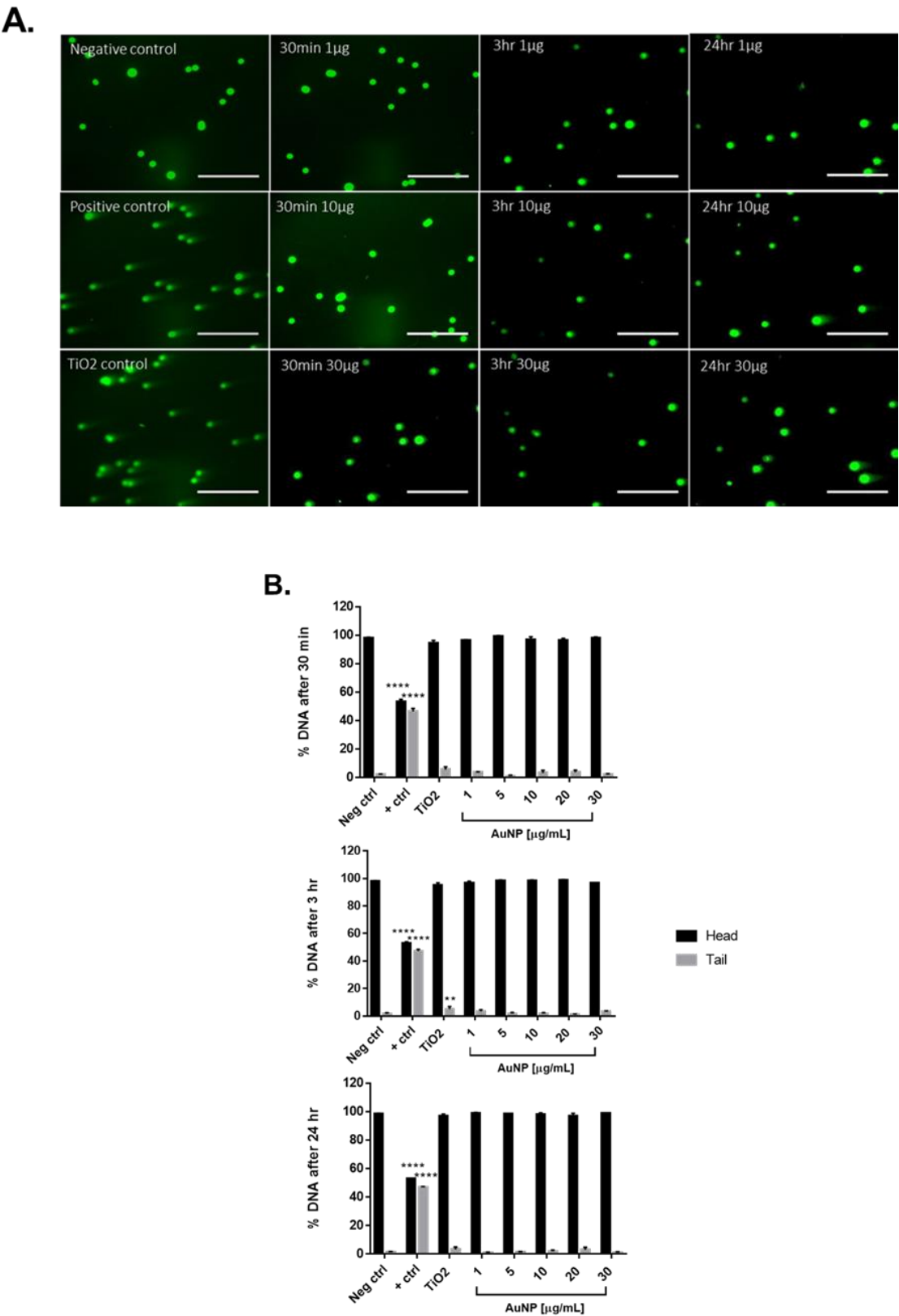
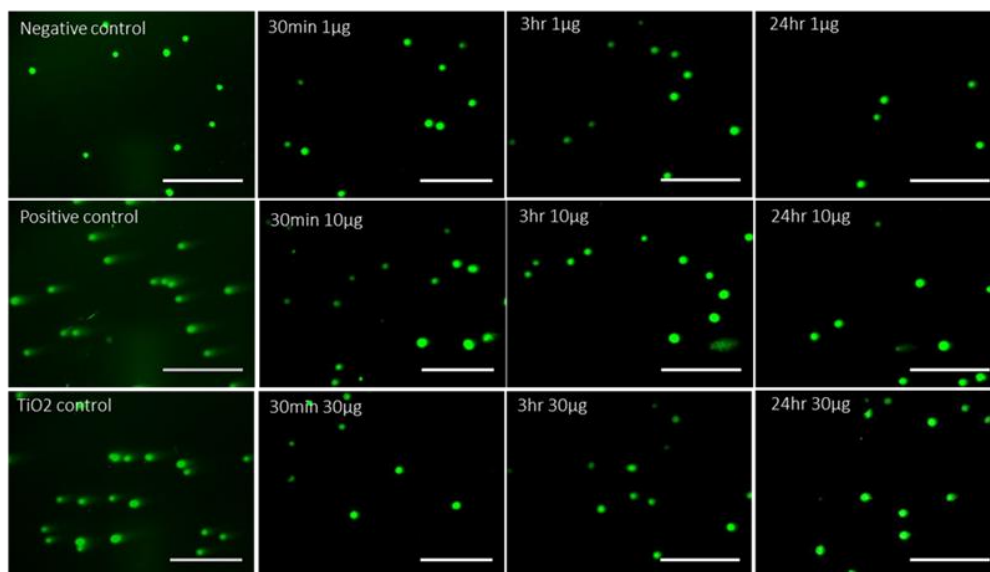


Figure 4.11. DNA damage induced by AuNP in 2D monolayer HepG2 cells assessed using the alkaline comet assay.

(A) Representative images of ethidium stained HepG2 cells treated with varying concentrations of AuNP over 30 min, 3 and 24h. (B) Percentage DNA head and tail values. Data representative of a minimum of three independent experiments ($n=3$) and expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.05$, **** $p < 0.0001$, statistical analysis by two-way ANOVA, followed by post-hoc Bonferroni test. Comets scored using the automated Comet IV software (Instem, United Kingdom) to avoid bias. (B) All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

A.



B.

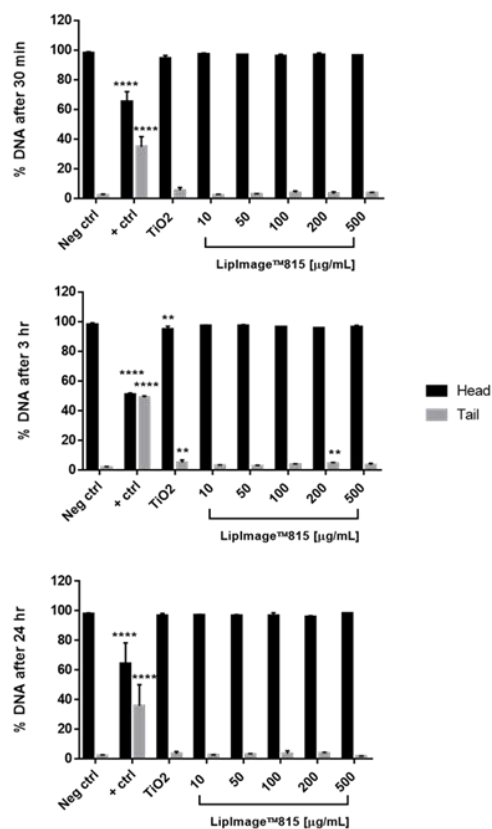


Figure 4.12. DNA damage induced by LipImage in HepG2 2D monolayers assessed using the alkaline comet assay.

(A) Representative images of ethidium stained HepG2 cells treated with varying concentrations of LipImage over 30 min, 3 and 24h. (B) Percentage DNA head and tail values. Data representative of a minimum of three independent experiments ($n=3$) and

*expressed as mean $\pm$ SEM. ** $p < 0.05$ and **** $p < 0.0001$, statistical analysis by two-way ANOVA, followed by post-hoc Bonferroni test. Comets scored using the automated Comet IV software (Instem, United Kingdom) to avoid bias. (B) All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).*

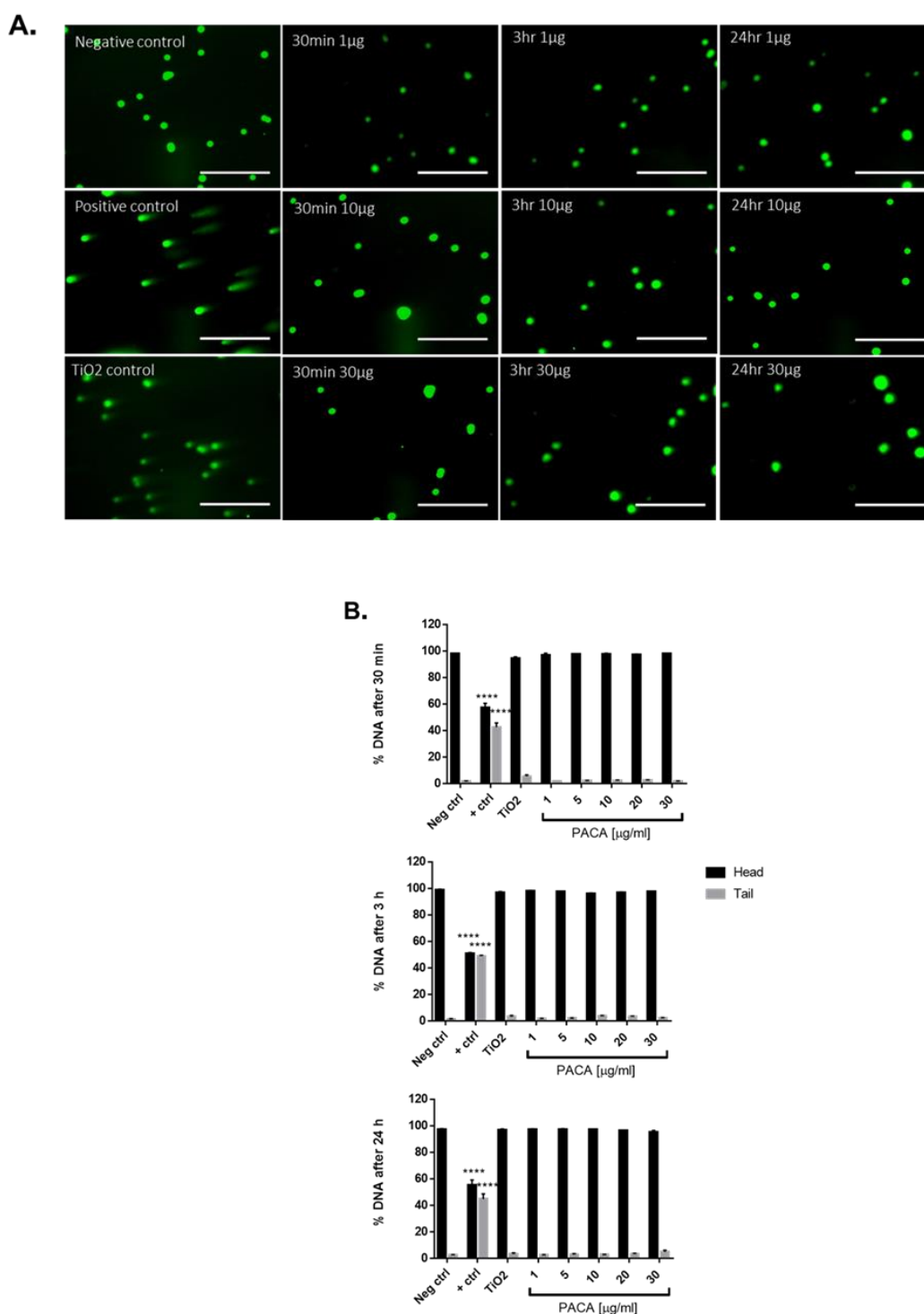


Figure 4.13. DNA damage induced by PACA in 2D monolayer HepG2 cells assessed using the alkaline comet assay.

(A) Representative images of ethidium stained HepG2 cells treated with varying concentrations of PACA over 30 min, 3 and 24h. (B) Percentage DNA head and tail values. Data representative of a minimum of three independent experiments ($n=3$) and expressed as mean $\pm$ SEM. **** $p < 0.0001$, Statistical analysis by two-way ANOVA, followed by post-hoc

Bonferroni test. Comets scored using the automated Comet IV software (Instem, United Kingdom) to avoid bias. (B) All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

4.3.4.2 Assessing NBM-induced DNA damage in monoculture and multicellular 3D spheroids using the alkaline comet assay

Using the adapted SOP for assessing NBM-induced DNA damage using the alkaline comet assay in HepG2 monolayers, DNA damage was also assessed in 3D hepatic monoculture spheroids. Due to the need to dissociate the hepatic spheroids prior to undertaking the alkaline comet assay, greater incidences of debris and cell aggregates were observed in the slide post-experiment, therefore great care was taken to score single cells only. In a similar manner to what was previously observed in 2D monolayers, both the EMS and TiO₂ positive controls induced significant DNA damage, with highest % tail value $35.92\% \pm 14.68$ (Figure 4.14, 4.15 and 4.16). Negative controls were also similar to results previously obtained, with % tail values of $1.45\% \pm 2.90$, $1.86\% \pm 2.52$ and $1.26\% \pm 2.02$ respectively. Each NBM screened induced low levels of genotoxicity, with % tail values for all treatments less than the 10% QC/QA acceptance criteria. Highest % of DNA damage induced by each NBM occurred following treatment with the largest concentration for AuNP and PACA (both 30 µg/ml), % tail values of $4.20\% \pm 1.20$ and $4.26\% \pm 0.70$ respectively. For LipImage, the 200 µg/ml induced the most DNA damage, with % tail value observed as $3.69\% \pm 0.80$. Significant differences were only observed in positive controls (Figure 4.14, 4.15 and 4.16). Assessing NBM-induced DNA damage was also trialed in HepG2/LX-2 multicellular spheroids, however in comparison to HepG2 spheroids alone, there was great difficulty in dissociating spheroids and the same protocols successfully used for dissociating HepG2 monoculture spheroids could not be successfully applied to the multicellular spheroids.

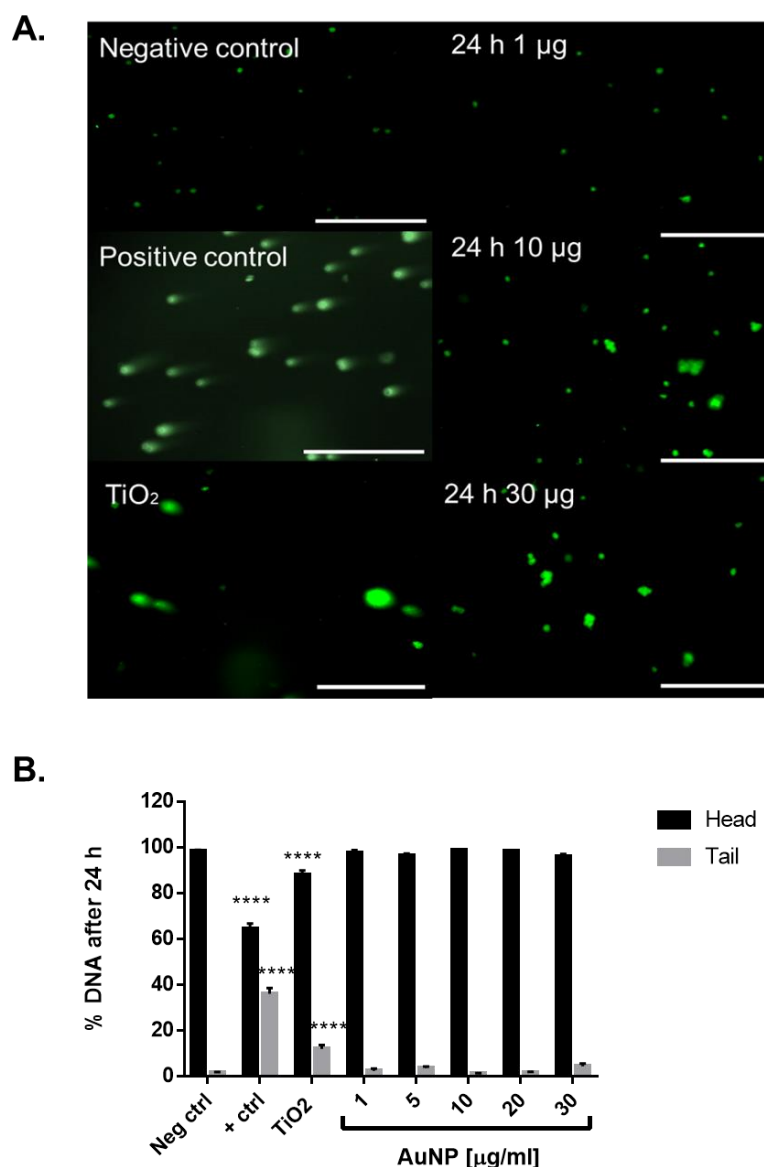


Figure 4.14. DNA damage induced in 3D HepG2 monoculture spheroids by AuNP.

(A) Representative images of ethidium stained HepG2 cells from dissociated 3D monoculture spheroids treated with AuNP 24h. (B) Percentage DNA head and tail values. Data representative of a minimum of three independent experiments ($n=3$), with 100 comets/cells scored per experiment. Data expressed as mean $\pm$ SEM. **** $p < 0.0001$, statistical analysis by Two-way ANOVA. Comets scored using the automated Comet IV software (Instem, United Kingdom) to avoid bias. (B) All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

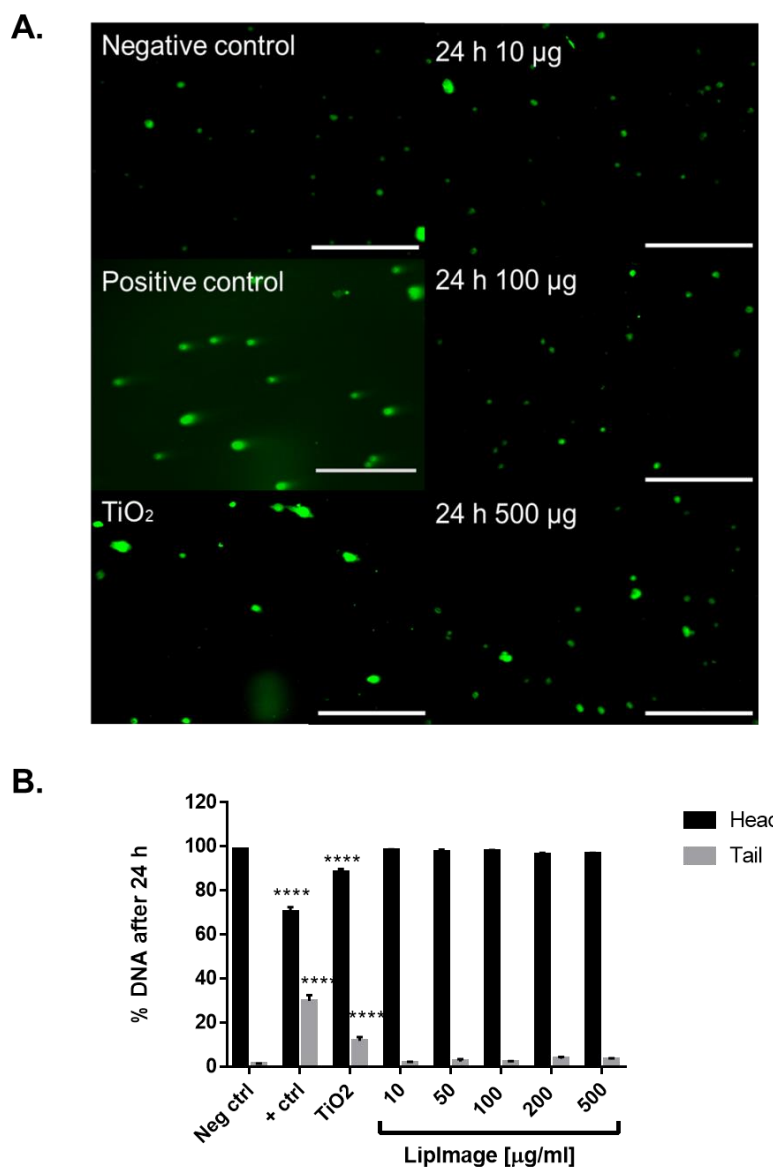
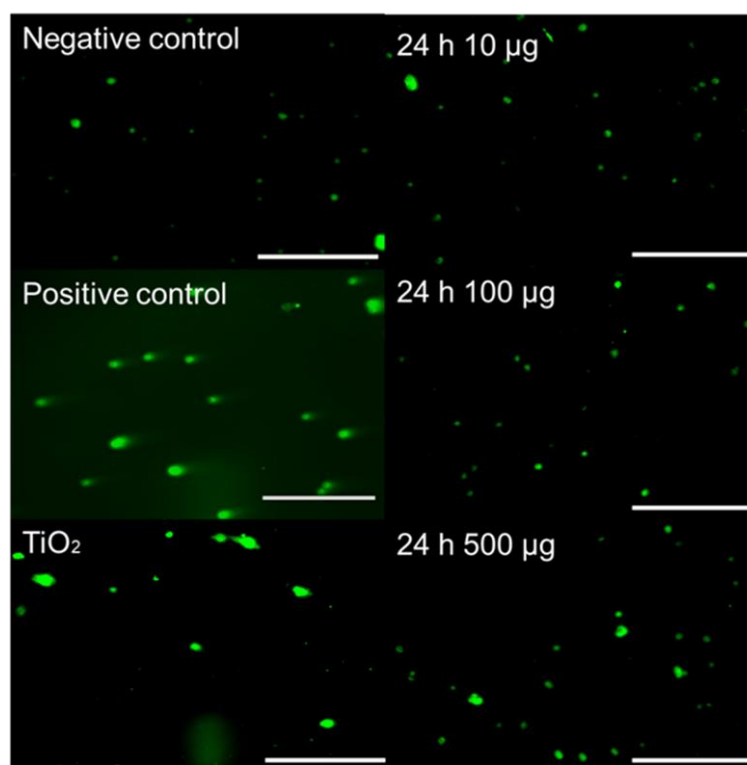


Figure 4.15. DNA damage induced in 3D HepG2 monoculture spheroids by LipImage.

(A) Representative images of ethidium stained HepG2 cells from dissociated 3D monoculture spheroids treated with LipImage for 24h. (B) Percentage DNA head and tail values. Data representative of a minimum of three independent experiments ($n=3$), with 100 comets/cells scored per experiment. Data expressed as mean $\pm$ SEM. **** $p < 0.0001$, statistical analysis by Two-way ANOVA. Comets scored using the automated Comet IV software (Instem, United Kingdom) to avoid bias. (B) All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

A.



B.

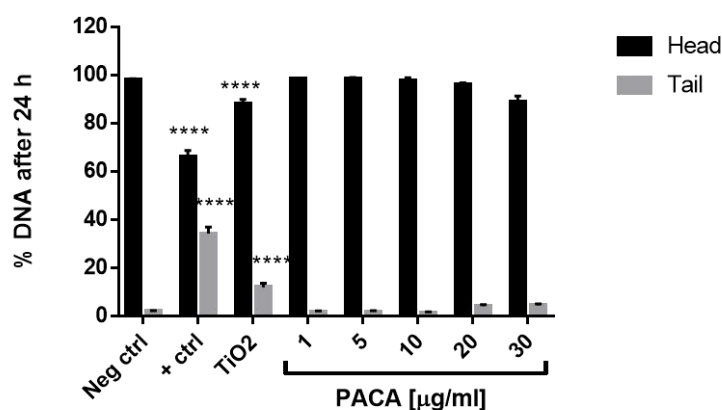


Figure 4.16 DNA damage induced in 3D HepG2 monoculture spheroids by PACA.

(A) Representative images of ethidium stained HepG2 cells from dissociated 3D monoculture spheroids treated with PACA for 24h. (B) Percentage DNA head and tail values. Data representative of a minimum of three independent experiments ($n=3$), with 100 comets/cells scored per experiment. Data expressed as mean $\pm$ SEM. **** $p < 0.0001$, statistical analysis by Two-way ANOVA. Comets scored using the automated Comet IV software (Instem, United Kingdom) to avoid bias. (B) All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

4.3.4.3 Comparing NBM-induced DNA damage in 2D monocultures and 3D monoculture spheroids

Comparison were then made between the 2D monoculture and 3D spheroid models. As observed in Figure 4.17, greater DNA damage was observed in spheroids cultures than 2D monolayers for many concentrations across each NBM. This was non-significant however, and significant differences were only observed between the genotoxic positive controls in each experiment.

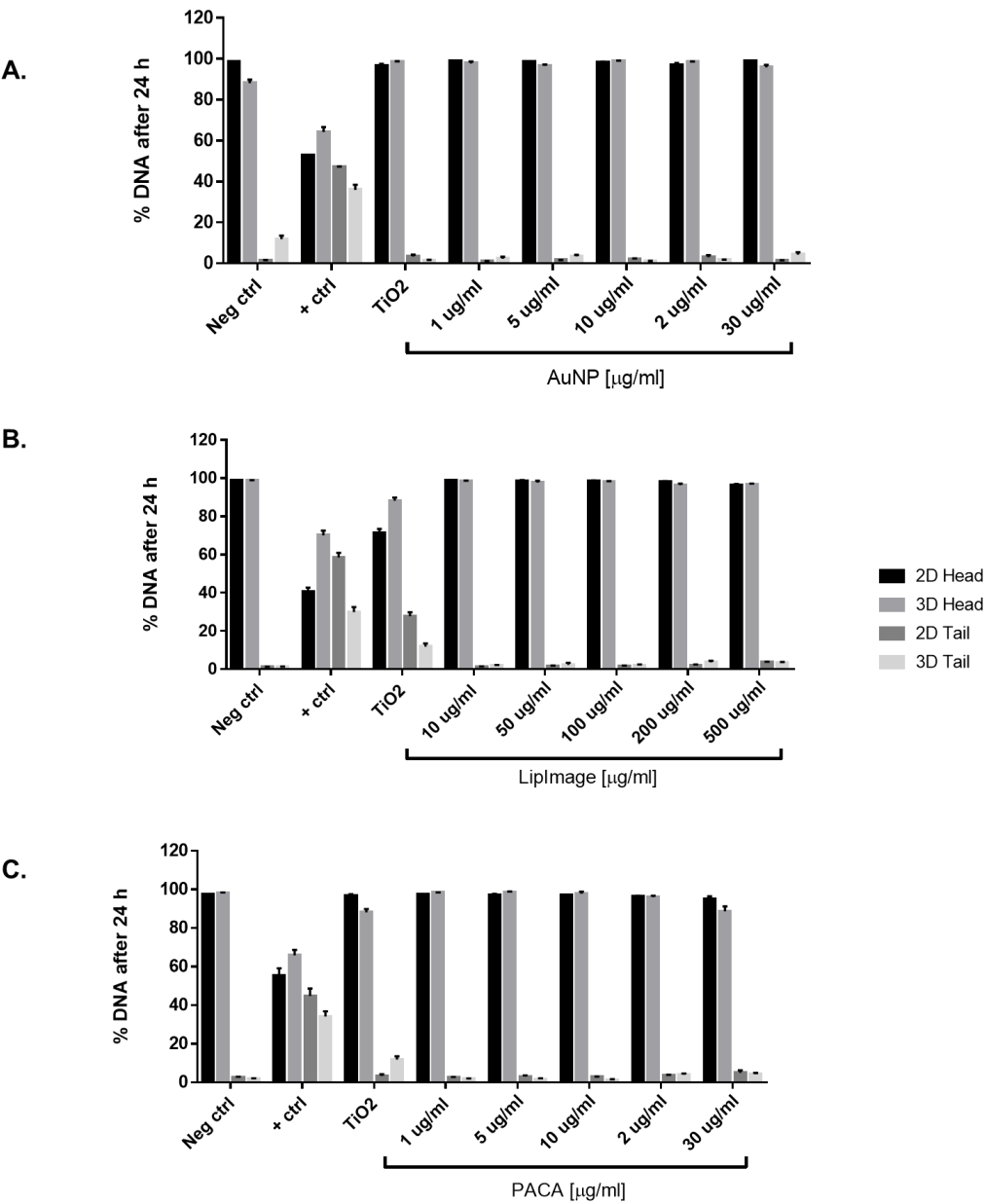


Figure 4.17. Comparison of DNA damage induced in HepG2 2D monocultures and 3D monoculture spheroids by (A) AuNP, (B) LipImage and (C) PACA.

Data representative of a minimum of three independent experiments (n=3), with 100 comets/cells scored per experiment. Statistical analysis by Two-way ANOVA presented below in Table 4.9. Comets scored using the automated Comet IV software (Instem, United Kingdom) to avoid bias. (B) All graphs and statistical analysis were undertaken using GraphPad Prism 7, Version 7.1.0 (GraphPad Software, Inc, San Diego, USA).

Table 4.7. % tail values for two-way ANOVA carried out on 2D and 3D cultures at each NBM concentration following 24 h incubation with each NBM.

Post hoc analysis carried out using Tukey method, *** < p0.001, ****p < 0.0001.

		<i>Neg-</i>	<i>EMS+</i>	<i>TiO2+</i>	<i>1</i>	<i>5</i>	<i>10</i>	<i>20</i>	<i>30</i>
<i>AuNP</i>	2D v. 3D	****	****	ns	ns	ns	ns	ns	ns
<i>PACA</i>	2D v. 3D	****	***	****	ns	ns	ns	ns	ns
		<i>Neg-</i>	<i>EMS+</i>	<i>TiO2+</i>	<i>10</i>	<i>50</i>	<i>100</i>	<i>200</i>	<i>500</i>
<i>LipImage</i>	2D v. 3D	****	****	****	ns	ns	ns	ns	ns

4.3.5 Validation of results obtained *via* the alkaline comet assay SOP using γ H2AX staining for double strand breaks

As a means of validating the SOP developed for assessing NBM-induced DNA damage using the alkaline comet assay, a second method of assessing genotoxicity was used. Phospho-histone 2AX (γ H2AX or pH2AX), a marker of double-strand DNA breaks, was assessed qualitatively using immunofluorescent and confocal imaging in 2D HepG2 monolayers and 3D monoculture and multicellular spheroids.

4.3.5.1 γ H2AX staining to assess NBM-induced DNA damage

As observed in Figure 4.18 (A), no double strand breaks (DSB) were seen in the negative control, in comparison to the EMS and TiO₂ positive controls (Figure 4.18 B and C), which both induced significant incidences of γ H2AX foci (indicative of DSB) in HepG2 monolayers. None of the NBM treatments, i.e. 24 h treatments with AuNP 30 μ g/ml, LipImage 500 μ g/ml, or PACA 30 μ g/ml, induced any visible foci inside the nuclei of HepG2 cells (Figure 4.18 D, E, F).

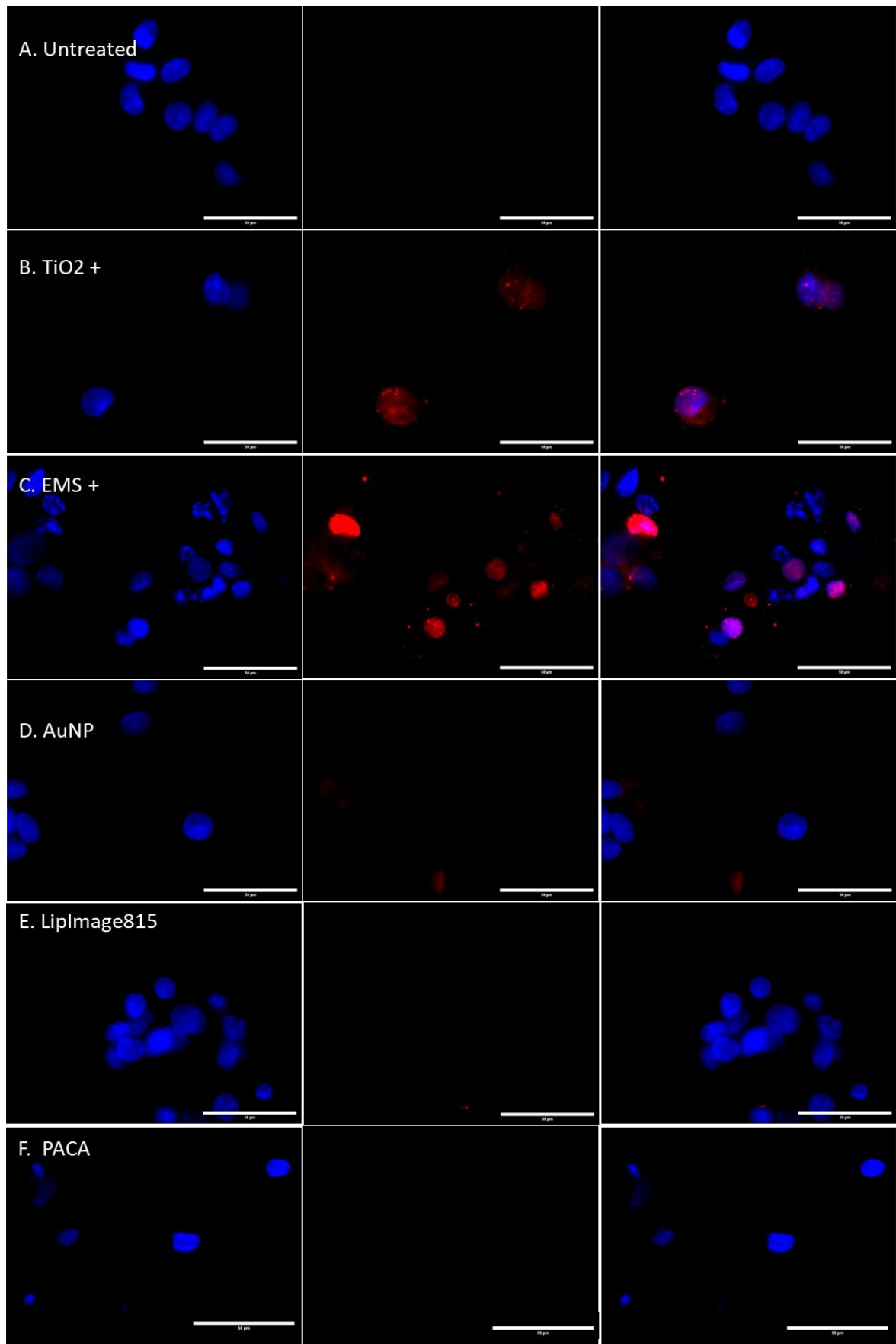


Figure 4.18. γ H2AX staining of 2D HepG2 monolayers following treatment with NBMs and controls.

(A) Untreated control, (B) TiO₂ positive control, (C) EMS positive control, (D) 30 µg/ml AuNP, (E) 500 µg/ml LipImage, and (F) 30 µg/ml NR668 PEBCA (PACA). Monolayers were treated with NBMs for 24 h and EMS positive control for 30 min before cells were fixed, permeabilised, blocked, and stained with γ H2AX Ab for 1h, followed by goat anti-mouse Alexa Fluor 532 Ab (red) and Hoechst (nuclei - blue). Imaging was undertaken using Epifluorescent microscopy (Nikon TE300) and 100X objective, with QCapture Software (QImaging). All image processing and measurements was undertaken using ImageJ software. Scale bar = 50 µm.

The presence of double strand breaks was then assessed in 3D hepatic monoculture and multicellular spheroids. As observed in Figure 4.19, spheroids treated with the EMS positive control induced large numbers of foci (indicated by white boxes), corresponding to the large induction of DNA damage from EMS assessed using the alkaline comet assay. Double strand breaks were also observed following treatment with TiO₂ positive control (Figure 4.20).

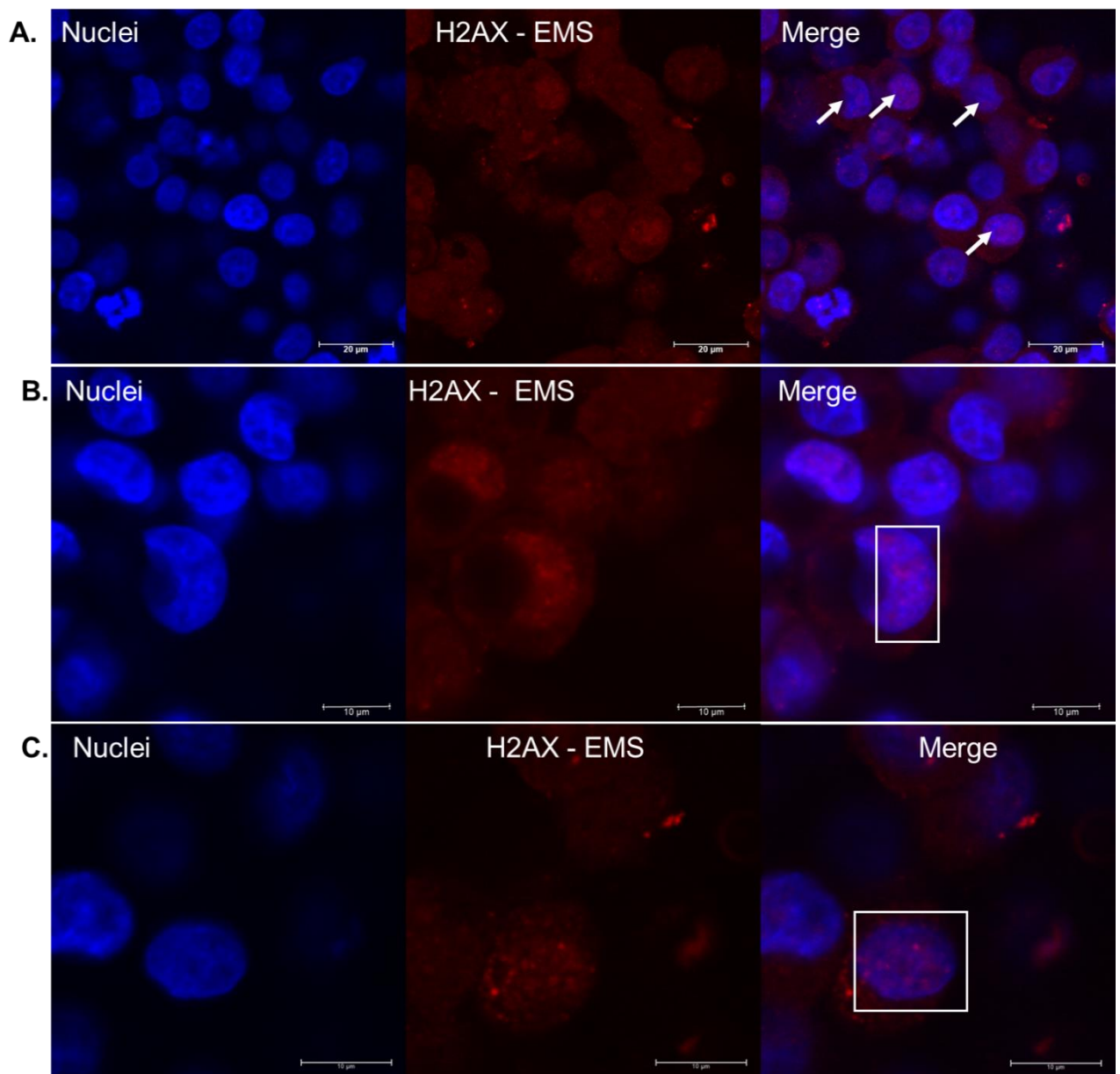


Figure 4.19. Double strand breaks (DSB) induced by EMS + control in 3D HepG2 spheroids assessed qualitatively via γ H2AX staining.

Representative images of spheroids grown for 8 d and treated with EMS positive control for 30 min before being fixed, permeabilised, blocked and stained with γ H2AX Ab for 1 h, followed by goat anti-mouse Alexa Fluor 532 Ab (red) and Hoechst (nuclei - blue). Foci, indicative of double strand breaks, are denoted by white boxes and arrows. Qualitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, TBSI, Trinity College Dublin, using a Leica system and 20 x magnification. Images processed using Leica LAS X software. Scale bars = (A) 20 μ m, (B) & (C) 10 μ m (zoomed in).

In comparison to the results obtained from the EMS positive control, the TiO₂ induced less DNA DSB, denoted by the presence of smaller numbers of foci, as observed in Figure 4.19.

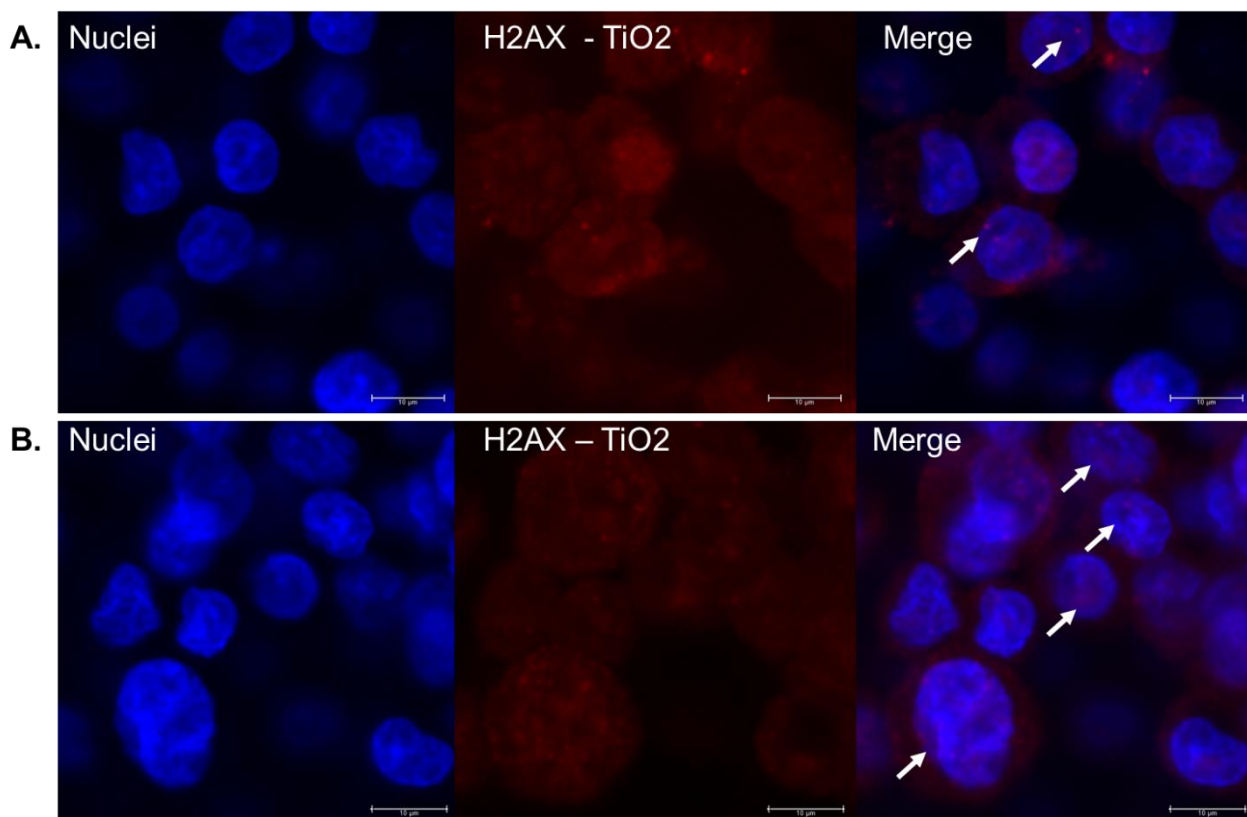


Figure 4.20. Double strand breaks (DSB) induced by TiO₂ + control in 3D HepG2 spheroids assessed qualitatively via γ H2AX staining.

Representative images of spheroids grown for 8 d and treated with TiO₂ positive control for 30 min before being fixed, permeabilised, blocked and stained with γ H2AX Ab for 1 h, followed by goat anti-mouse Alexa Fluor 532 Ab (red) and Hoechst (nuclei - blue). Foci indicated by white arrows. Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, TBSI, Trinity College Dublin, using a Leica system and 20 x magnification. Images processed using Leica LAS X software. Scale bars = 10 μ m.

The first NBM assessed in spheroids using γ H2AX staining was AuNP. As observed in Figure 4.21, a small number of foci is observed following 24 h treatment with 30 μ g/ml AuNP. This corresponds to similar results obtained using the alkaline comet assay, whereby this AuNP treatment did not induce any significant genotoxicity, i.e. a % DNA in tail value of $1.30\% \pm 0.39$. (Figure 4.14).

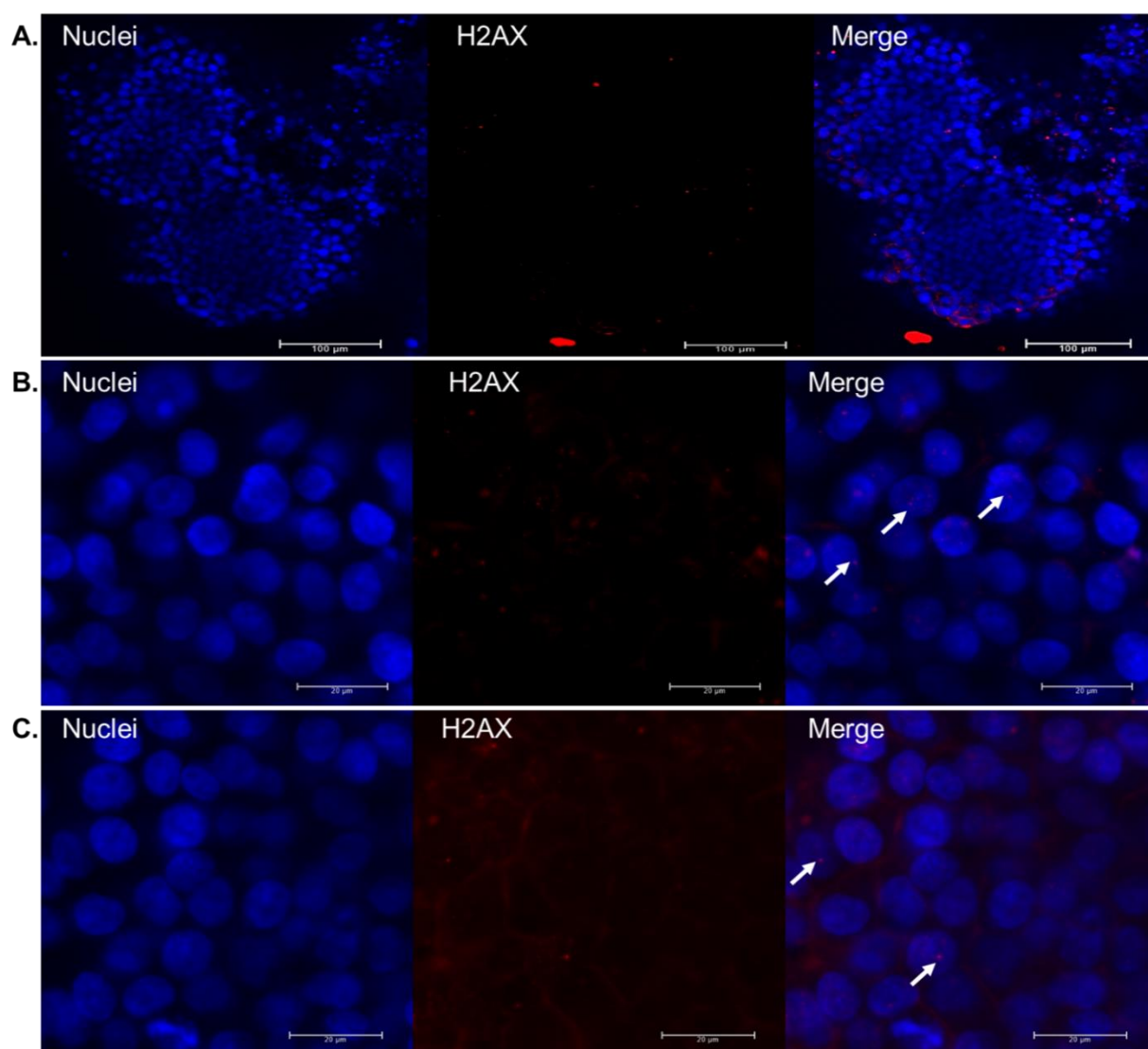


Figure 4.21. Double strand breaks (DSB) induced by 30 $\mu\text{g/ml}$ AuNP in 3D spheroids, qualitatively assessed via γH2AX staining

Representative images of HepG2 monoclulture spheroids grown for 8 d and treated with 30 $\mu\text{g/ml}$ AuNP for 24 h before being fixed, permeabilised, blocked and stained with γH2AX Ab for 1 h, followed by goat anti-mouse Alexa Fluor 532 Ab (red) and Hoechst (nuclei - blue). Foci indicated by white arrows. Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, TBSI, Trinity College Dublin, using a Leica system and 20 x magnification. Images processed using Leica LAS X software. Scale bars = (A) 100 μm , (B) & (C) 20 μm (zoomed in).

3D HepG2 monoclulture spheroids were then treated with LipImage before being assessed using γH2AX staining. As observed in Figure 4.22, no distinct foci are observed following 24 h treatment with 500 $\mu\text{g/ml}$ AuNP. This corresponds to similar results obtained using the

alkaline comet assay, whereby this LipImage treatment did not induce any significant genotoxicity, i.e. a % DNA in tail value of $1.09 \% \pm 0.39$. (Figure 4.15).

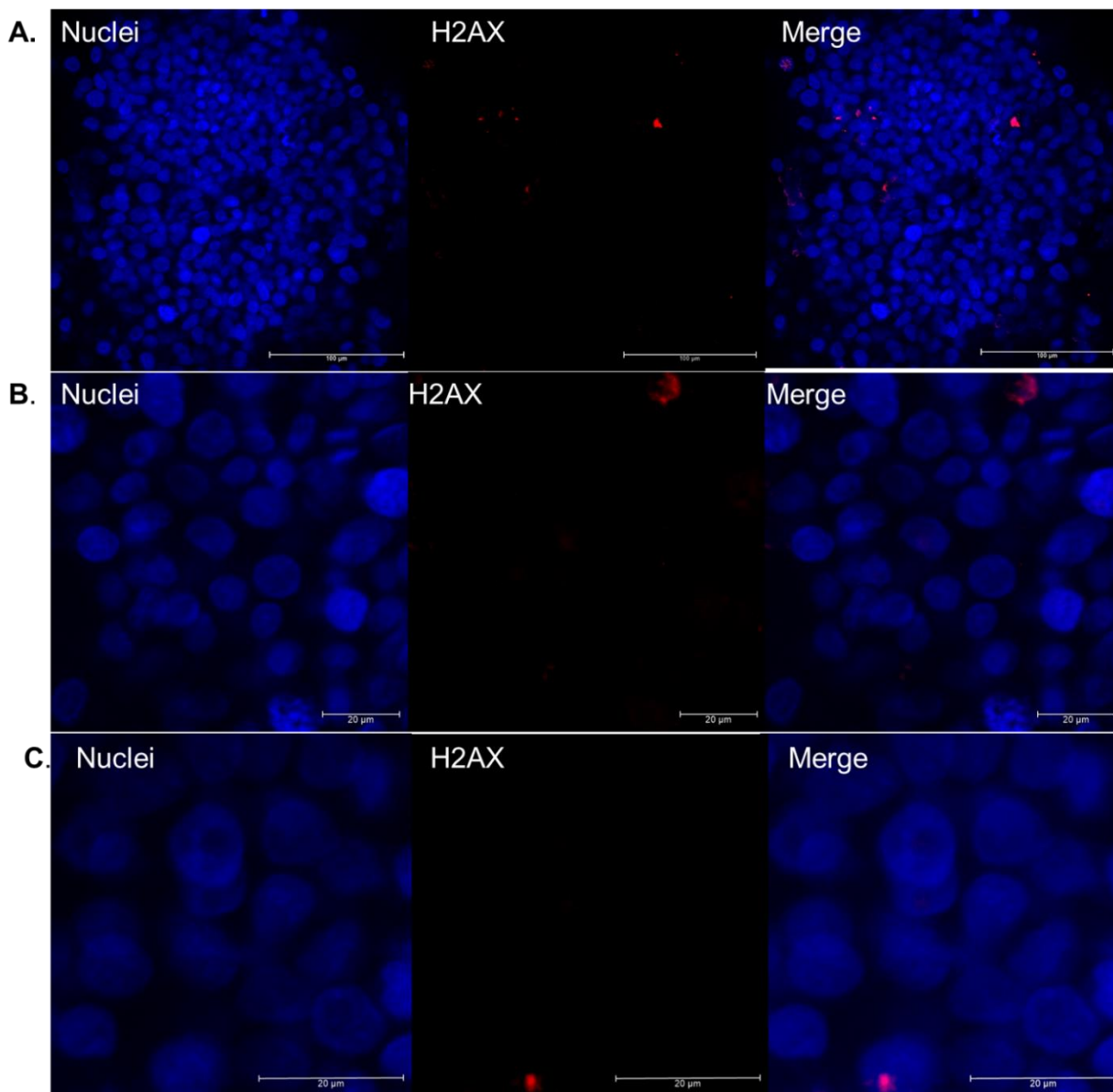


Figure 4.22. Double strand breaks (DSB) induced by 500 µg/ml LipImage in 3D spheroids qualitatively assessed via γ H2AX staining.

Representative images of HepG2 monoclulture spheroids grown for 8 d and treated with 500 µg/ml LipImage for 24 h before being fixed, permeabilised, blocked and stained with γ H2AX Ab for 1 h, followed by goat anti-mouse Alexa Fluor 532 Ab (red) and Hoechst (nuclei - blue). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, TBSI, Trinity College Dublin, using a Leica system and 20 x magnification.

Images processed using Leica LAS X software. Scale bars = (A) 100 μ m, (B) & (C) 20 μ m (zoomed in).

3D HepG2 monoculture spheroids were then treated with the final NBM, PACA, before being assessed using γ H2AX staining. As observed in Figure 4.23, small numbers of foci are observed following 24 h treatment with 30 μ g/ml PACA. This is also in concordance to similar results obtained previously using the alkaline comet assay, whereby this PACA treatment induced levels of DNA damage below the acceptance criteria, any significant genotoxicity, i.e. a % DNA in tail value of $1.09\% \pm 0.39$. (Figure 4.16).

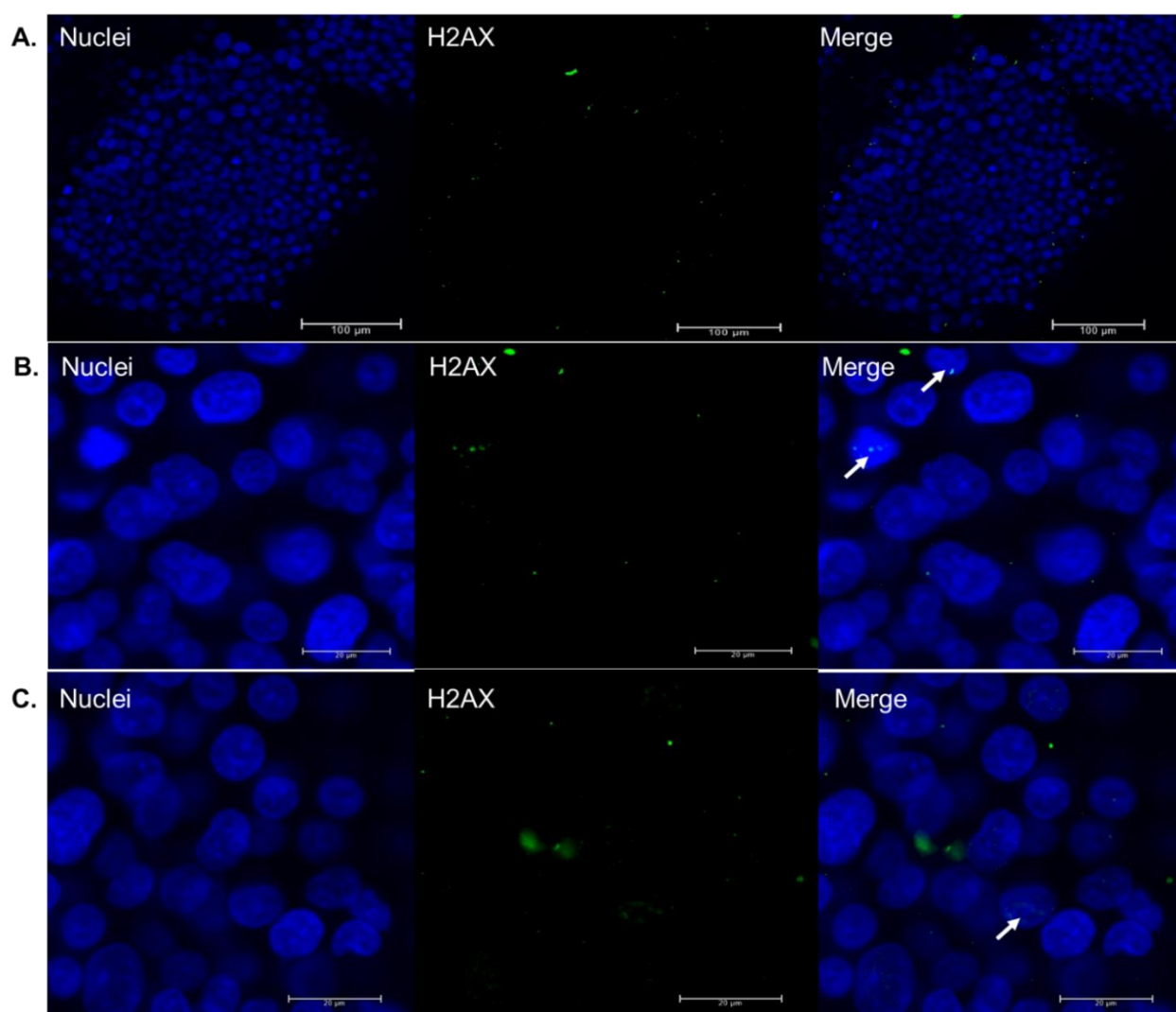


Figure 4.23. Double strand breaks (DSB) induced by 30 μ g/ml PACA in 3D spheroids qualitatively assessed via γ H2AX staining.

Representative images of spheroids grown for 8 d and treated with 500 µg/ml LipImage for 24 h before being fixed, permeabilised, blocked and stained with γH2AX Ab for 1 h, followed by goat anti-mouse Alexa Fluor 488 Ab (green) and Hoechst (nuclei - blue). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, TBSI, Trinity College Dublin, using a Leica system and 20 x magnification. Images processed using Leica LAS X software. Scale bars = (A) 100 µm, (B) & (C) 20 µm (zoomed in).

As it was not possible to assess the extent the chosen NBMs induced DNA damage using the alkaline comet assay, due to previously stated issues with dissociating these more compact spheroids, γH2AX staining was undertaken to qualitatively assess genotoxicity in 3D HepG2/LX-2 multicellular spheroids. As observed in Figure 4.24 and in a similar manner to the 3D HepG2 monoculture spheroids (Figure 4.21), small numbers of foci, indicated by white arrows, can be observed following 24 h incubation with AuNP. After incubation for 24 h with 500 µg/ml LipImage, small numbers of foci can be observed (Figure 4.25), in comparison to the 3D HepG2 monoculture spheroids where no visible foci can be observed (Figure 4.22). Following treatment with PACA (30 µg/ml for 24 h), no visible foci can be observed (Figure 4.26), in comparison to the 3D HepG2 monoculture spheroids where small numbers of foci are visible (Figure 4.23).

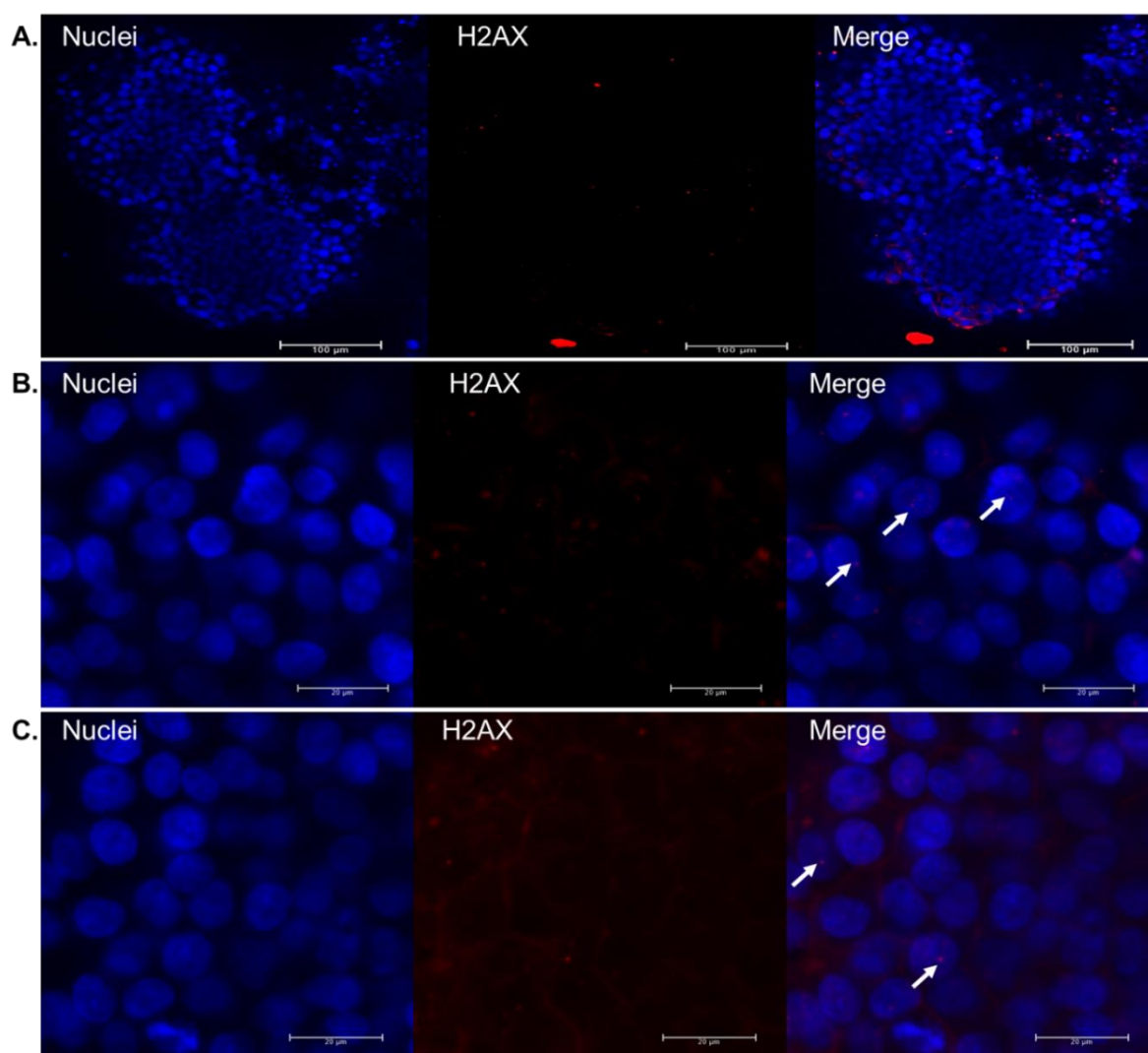


Figure 4.24. Double strand breaks (DSB) induced by 30 $\mu\text{g/ml}$ AuNP in 3D HepG2/LX-2 multicellular spheroids assessed via γH2AX staining.

Representative images of multicellular spheroids cultured for 8 d and treated with 30 $\mu\text{g/ml}$ PACA for 24h before being fixed, permeabilised, blocked and stained with γH2AX Ab for 1 h, followed by goat anti-mouse Alexa Fluor 532 Ab (red) and Hoechst (nuclei - blue). Qualitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, TBSI, Trinity College Dublin, using a Leica system and 20 x magnification. Images processed using Leica LAS X software. Scale bars = (A) 100 μm , (B) & (C) 20 μm (zoomed in).

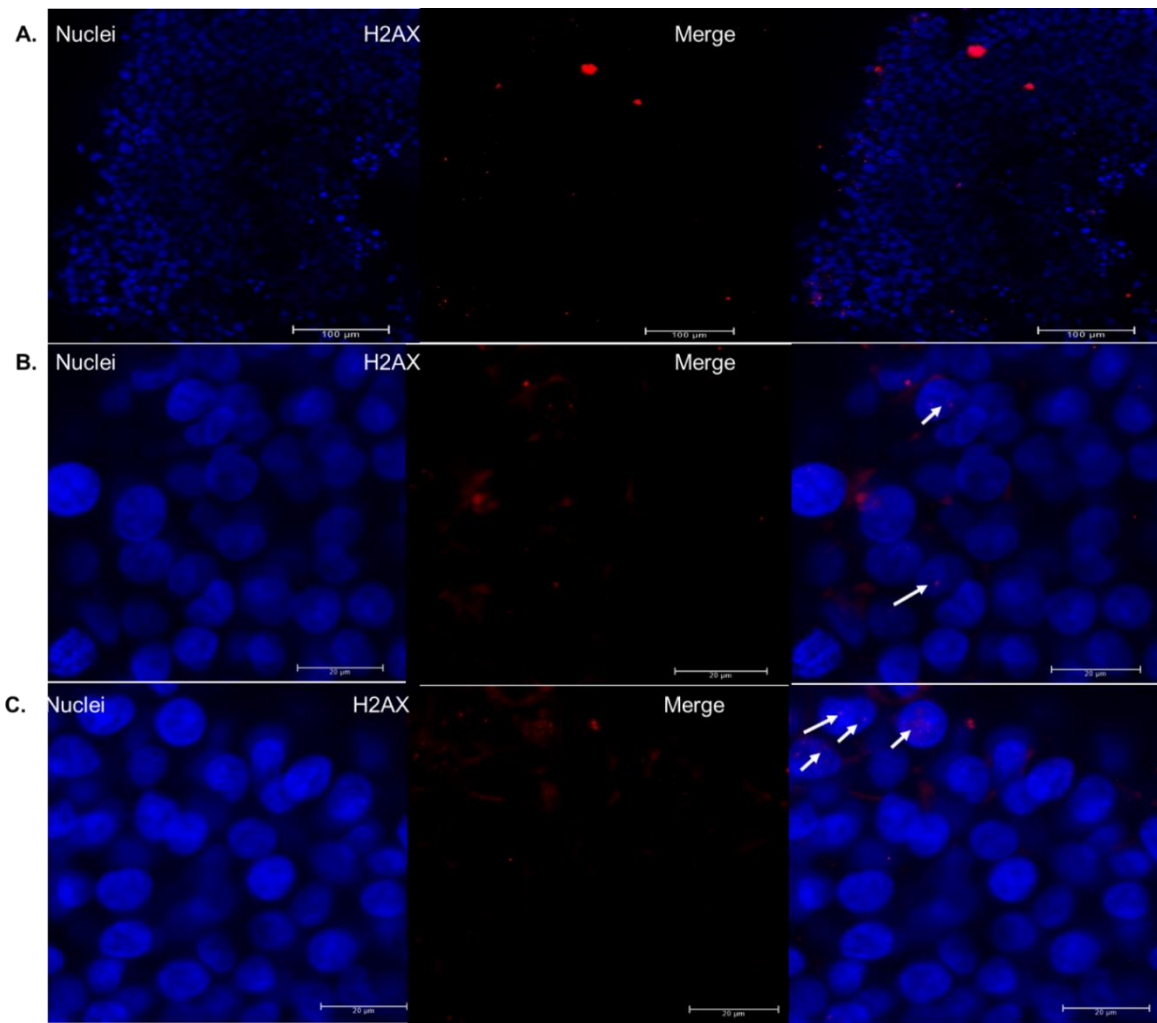


Figure 4.25. Double strand breaks (DSB) induced by 500 $\mu\text{g/ml}$ LipImage in 3D HepG2/LX-2 multicellular spheroids assessed via γH2AX staining.

Representative images of multicellular spheroids were cultured for 8 d were treated with 500 $\mu\text{g/ml}$ LipImage for 24h before being fixed, permeabilised, blocked and stained with γH2AX Ab for 1h, followed by goat anti-mouse Alexa Fluor 532 Ab (red) and Hoechst (nuclei - blue). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, TBSI, Trinity College Dublin, using a Leica system and 20 x magnification. Images processed using Leica LAS X software. Scale bars = (A) 100 μm , (B) & (C) 20 μm (zoomed in).

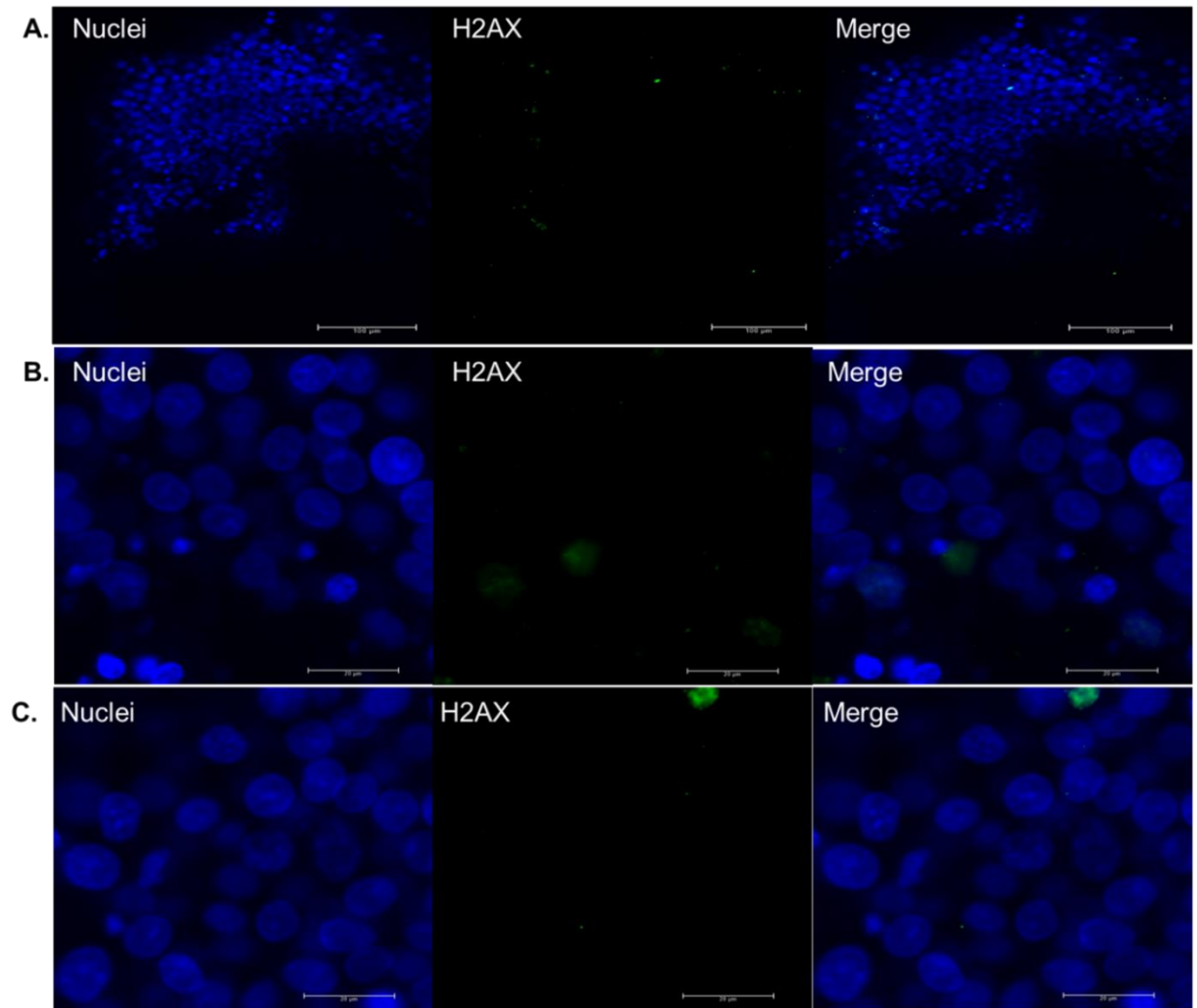


Figure 4.26. Double strand breaks (DSB) induced by 30 µg/ml PACA in 3D HepG2/LX-2 multicellular spheroids assessed via γ H2AX staining.

Representative images of multicellular spheroids, cultured for 8 d were treated with 30 µg/ml PACA for 24h before being fixed, permeabilised, blocked and stained with γ H2AX Ab for 1h, followed by goat anti-mouse Alexa Fluor 532 Ab (red) and Hoechst (nuclei - blue). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, TBSI, Trinity College Dublin, using a Leica system and 20 x magnification. Images processed using Leica LAS X software. Scale bars = (A) 100 µm, (B) & (C) 20 µm (zoomed in).

4.4 Discussion

In recent decades, due to the development and production of various NBMs with many novel properties and applications, concerns have been raised over their potential to impact human health [402, 403]. From their broad uses day to day ranging from pigments in food and cosmetics to their antimicrobial properties and ability to act as drug carriers or tracers, we are likely to become exposed to NPs or NBMs, either intentionally, occupationally or accidentally [284, 404-406]. Many *in vivo* studies have demonstrated the strong accumulation and off-target side effects of NBMs in the liver, a primary organ of interest regarding NBMs and various other xenobiotics due to its role in detoxification [407], and today, induction of hepatotoxicity is one of the predominant reasons that a medicine may be rejected or removed from the market [408]. Because of this, there is a pressing need for more sensitive and *in vivo*-like hepatotoxicity screening methods which can be used during the development and assessment of NBMs. Although *in vivo* toxicity is a regulatory requirement and must be assessed during the pre-clinical assessment of NBMs, vast amounts of both time and money can be saved if toxic effects can be determined early in the R&D process [409], and despite the fact *in vitro* cytotoxicity cannot be directly correlated to *in vivo* experimentation, it is generally regarded as an efficient and cost-effective initial measure of NBM toxicity [410, 411].

To address the bottleneck commonly seen in the NBM drug discovery process, in recent decades 3D *in vitro* models have emerged as useful platforms which may be used in conjunction with traditional models for screening toxicity and efficacy of new therapeutics [412, 413]. One of the most used 3D *in vitro* model today, and the focus of this thesis, is 3D hepatic spheroids [167]. The use of 3D spheroids for assessment of NBM-induced hepatotoxicity has, in recent years, also become a growing area of interest [283, 286, 414]. Despite providing valuable information, 2D cell culture models do not accurately predict *in vivo* toxicity and other biological effects of NBMs, due to the absence of key physiological processes [415], including transport of NBMs through varying cell layers when they come in contact with tissues [223]. In combination with this, with regard to cellular function, essential effects of NBMs which might be strongly dependent on 3D organization are neglected. Possibly the most important factor to discuss, and the most substantiating evidence to suggest expanding toxicity assessment to 3D, is the large discrepancies observed between toxicity results from 2D *in vitro* cell culture assays and *in vivo* animal models [416]. Recent studies undertaken in 2D culture for the toxicity screening of various magnetic NBMs [417], carbon nanotubes [418] and quantum dots [419] have exhibited high cytotoxic

effects, however when tested in *in vitro* animal models, no adverse effects were observed [105, 420-422]. Issues regarding unintended hepatotoxicity are not limited to iron oxide nanoparticles, with various other NBM formulations exhibiting similar issues. For example, AmBisome[®], the liposome form of amphotericin B, has demonstrated non-dose dependent deranged liver function *in vivo*, with an increase in bilirubin and transaminases [423], issues not noted *in vitro*. As a means of overcoming these discrepancies and enhancing the predictive power of current *in vitro* toxicity screening methods, *in vitro* 3D cell culture has been introduced to bring the gap between traditional 2D models and *in vivo* animal models and for estimating *in vivo* toxicity. The difference in toxicity observed between 2D and 3D cultures is not yet fully clear however, and much work has yet to be done to fully underpin the differences between the two culture methods [284].

In this study, three NBM candidates for medical applications were assessed, each with differing physical characteristics. In recent years, huge efforts have been placed on designing and developing new NBMs which not only can increase the delivery of their encapsulated material or drug to cancer cells, but also facilitate greater therapeutic efficacy, and more sensitive, specific imaging [409]. However, whilst the use of spheroids in the study of NBMs has increased in the previous decade, efforts have predominantly focused on assessing the safety of use for large scale manufactured nanomaterials, for example ZnO, Ag and TiO₂ which are produced in tonnages. NBMs for medical technologies, or those featured in this study [284] are still limited in use and manufactured production but are fast grown in their uses and application range.

A wide array of *in vitro* screening methods can be applied to NBMs, however it is vitally important to use an appropriate assay for the specific NBM being tested. A significant challenge met when assessing NBMs is particle interference. Many *in vitro* assays have readout parameters that are based on transmission, i.e., plate readers that measure quantity of transmitted light at various wavelengths, with this measure translated to optical density. These systems are also predominantly designed for testing materials which are transparent, such as culture media and dilute cell lysates. This same transparency is not observed for many NBMs, which are often very optically dense, and whilst NBMs normally do not emit light, they can absorb it, therefore greatly impacting readouts from many *in vitro* assays that are classed as standard. Particle density depends on a variety of factors including particle type, e.g., gold absorbs much greater light than iron oxide NPs, size, with larger NPs absorbing more light than smaller ones, and concentrations tested. NP, or also NBM, density can cause increased readouts, leading to overestimations of optical density and inaccurate results regarding viability, toxicity etc [424]. This issue is most often seen with the MTT

assay, a widely used cytotoxicity assay which can determine cellular viability or metabolic activity by measuring the ability of active cells to transform the water-soluble dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into an insoluble formazan (indicative of number of living cells), which can then be detected using a luminescently. Results from the MTT assay are significant skewed by many NPs giving a false impression of increased proliferation and improved viability [366-368]. On the other hand, when toxic NPs are screening using MTT, decreased formazan production, which occurs due to reduced cell metabolism, may be masked by the optical density of the NP in question, providing false information of lack of toxicity. Some of the most widely used NPs today, including many small NPs like Ag, Au, CoO, Fe₃O₄ and CeO₂ all absorb at wavelengths which are most used biological assay readouts, i.e., 340, 380, 405, 440, 540/550 nm [424].

Another key consideration is the suitability of the chosen assay for use with cell spheroids and not just monolayers. Many standard colorimetric *in vitro* assays based on tetrazolium reduction, i.e., MTT, or reduction of resazurin (Alamar blue assay) have been deemed unsuitable for 3D spheroids or scaffold cultures in general, due to the tight cell-cell junctions and *in vivo* like architecture which affects uptake and diffusion kinetics of the respective dyes, therefore altering readouts of the assays and making results obtained difficult to interpret [425-427]. To overcome some of these issues, which are common to also the emerging development of NBMs, in this study the CellTiter-Glo® ATP quantification, a frequently used assay for 3D microtissues, was used for determining 3D spheroid viability. Here, 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 [425]. 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 [428].

The third and final issue that was assessed was NBM-induced DNA damage, or genotoxicity. Genotoxicity is described as the potential of NBMs to potentially damage the genetic material of the nucleus, with NBM-induced genotoxicity being either primary, defined by the impact of the materials themselves and not inflammatory response, or secondary, whereby involvement of inflammatory cells causes oxidative DNA damage via ROS [429].

Despite the OECD providing many useful recommendations on *in vitro* genotoxicity testing of NBMs, unlike uptake studies, cytotoxicity, immunotoxicity, toxicology and haematology [71], NBM-induced genotoxicity is a largely overlooked area, and to date until this study, no standardised or validated SOP for assessing NBM-induced genotoxicity existed. The comet assay in the past has been successfully reported for NBM genotoxicity evaluation, and in this study the protocol for the alkaline comet assay was adapted for use specifically with NBMs [430]. An automated comet scoring software, Comet IV (Instem, United Kingdom), was used in this study, and is recommended as part in the SOP, in order to remove any subjectivity or bias when scoring comets. As outcomes of genotoxicity assays have been proved to vary significantly between groups in intra- and inter-laboratory comparisons, a developed SOP for the comet assay was tested robustly in an interlaboratory comparison study (Data provided in Supplementary Figure S4.6), as part of the EC H2020 REFINE project. An external round robin, starting in January 2021 will act as further validation for this protocol. Due to the fact the comet assay is slightly limited due to potential interference with induced cytotoxicity following NBM exposures (only non-cytotoxic concentrations may be used), the comet assay must be used in combination with other cytotoxic evaluation methods to ensure genotoxicity is related to concentrations above 80% viability [105, 428, 431].

A second genotoxicity assay may also be used to validate results, for example γ H2AX staining. For decades, γ H2AX staining has been widely used for both visualizing and quantifying radiation-induced double strand breaks (DSBs) in DNA [432-435], with the phosphorylation of serine 139 of H2AX being one of the first cellular responses to ionizing radiation [436, 437]. Various studies have demonstrated the close relationship between DSB and γ H2AX foci [432-434, 438, 439]. γ H2AX staining may also provide a higher sensitivity to DNA damage than the comet assay, despite being limited by not being able to provide information about mechanisms of DNA damage. From a regulatory perspective, genotoxicity screening requires a battery of tests addressing many different endpoints, therefore γ H2AX staining was included in this study as a second genotoxic endpoint [440]. To represent a metallic NBM, a benchmark 20nm PEGylated AuNP was the first NBM screened in this study. Despite being generally regarded as safe, literature regarding AuNP induced toxicity is conflicting, and it has been suggested that AuNPs may still cause off-target side effects, stimulating inflammatory responses and accelerate stress-induced apoptosis in the liver [425-427]. Exposure and accumulation to PEG-AuNPs in the RES and MPS in the liver has also been shown to expose hepatic tissues to delayed chronic low-grade inflammation [384]. Some studies have found that AuNPs can be toxic, with this toxicity

highly dependent on size and surface characteristics [441]. Goodman *et al.* have found that cationic gold nanospheres of 2 nm in diameter are toxic at specific doses. Interestingly, the same AuNPs were found to be non-toxic at the same concentration provided they had negatively charged surfaces [442]. 1.4 nm AuNPs spheres have been shown to trigger mitochondrial damage, cause necrosis and mitochondrial damage, and induce oxidative stress on epithelial and endothelial cells, fibroblasts and phagocytes, as shown by Pan *et al.*, who also found that there was no evidence of the same cellular damage for 15 nm AuNP nanospheres [443]. This highlights a key trend observed with toxicity screening of AuNPs – size dependent toxicity. Furthermore, whilst PEG coatings have been shown to improve AuNP distribution via blood circulation, their use on AuNPs has been shown to result in acute liver injury, via NF-KB induction, with induction dependent once more on PEG-AuNP size and surface modification [384]. Whilst some studies have assessed the uptake and interaction of varying sized AuNPs in spheroid models [444, 445], with others using hepatic spheroids to assess AuNP-induced cytotoxicity [446, 447], to the best of my knowledge HepG2 monoculture and multicellular spheroids have not been used to assess 20nm AuNP toxicity previously. Regarding AuNP induced genotoxicity, similar trends are observed, with increased DNA damage occurring in a time and concentration dependent manner. Xia *et al.* have found once again that smaller AuNPs, i.e., 5 nm, are more toxic than their larger (20nm and 50nm) counterparts, inducing more DNA damage in HepG2 cells in the Comet assay [448]. Wang *et al.* have published an extensive review on the genotoxic potential of AuNPs, citing no less than 13 *in vitro* experiments, including the comet assay, the Ames test, the micronucleus (MN) assay, the chromosome aberration (CA) assay, and Liquid chromatography/tandem mass spectrometry (LC/MS/MS) in various cell lines including CHO, HepG2, BALB/c 3T3, and HL-60 cells among others. Once again, it is reported that to the most part, AuNP-induced DNA damage occurs in a concentration, time and particle size dependent manner. This review cites the importance of using a barrage of genotoxicity endpoints to study genotoxic potential of NBMs, and the importance of reporting fully physicochemical characteristics of the target NBMs to avoid inconsistencies between studies, and suggests that the comet assay, the assay used in this thesis, should be preferentially applied to AuNP genotoxicity screening [449]. Regarding *in vivo* studies of AuNPs, similar contradicting data is presented regarding toxicity. Zhang *et al.* have undertaken *in vivo* toxicity screening of 5, 10, 30, and 60 nm PEG-coated gold nanoparticles in mice [450]. Here, no obvious decrease in body weight or appreciable toxicity was observed after their breakdown *in vivo*. 10m and 60 nm AuNPs however did cause a significant increase in aspartate transaminase (ATT) and alanine transaminase (ALT),

indicative of slight liver damage. Chen *et al.* have assessed the biodistribution, organ toxicity and changes in inflammatory cytokines in mice after exposure to 21 nm AuNPs [451], finding no observable changes in macrophages, and no detectable toxicity in vital organs such as the liver and kidney. Zhang *et al.* have assessed the biodistribution and pharmacokinetics of various sized PEG AuNPs, namely 20, 40 and 80 nm, in nude mice [452], finding that the 20nm AuNPs exhibited the slowest clearance from the body, with the highest uptake in tumours. They showed significant blood pool activity when compared to their larger counterparts but exhibited less uptake in liver and spleen.

With the above being said, in this thesis HepG2 monocultures, 3D spheroids and multicellular spheroids were treated with increasing concentrations of AuNP before being assessed using the assays previously detailed in order to determine if hepatic spheroids would be better predictors of AuNP hepatotoxicity than 2D monoculture. Evaluation of cell viability was undertaken first, using an ATP quantification assay. Levels of ATP remained constant in HepG2 monolayers and spheroids after AuNP exposure. At the highest concentration, 30 µg/ml, % viability in HepG2 monolayers was $89\% \pm 4.15$, $89\% \pm 3.62$ and $86\% \pm 2.73$ after 24, 48 and 72h respectively (Figure 4.4A). When compared to HepG2 monolayers, as expected, both HepG2 monoculture spheroids and HepG2/LX-2 multicellular spheroids exhibited a greater % viability than 2D monolayer cultures following 72 h incubations. Statistical analysis revealed however there was no significant drop in % viability at any of the chosen AuNP concentration and time point for both HepG2 monolayers and monoculture 3D spheroids (Table 4.2). The only significant difference was observed after 24h treatment between 2D monolayers and 3D multicellular spheroids that had been treated with 1 and 5 µg/ml (p value 0.004 and 0.005 respectively). It can be hypothesised that the increased viability in the 3D spheroid models is due to the fact spheroid cultures are, in a similar manner to *in vivo* liver tissue, less susceptible to cell death than monolayer cultures, where all cells in flat monolayers come in contact with NBMs. After assessing the viability of the various models, the ability of AuNPs to induce cytotoxicity was then assessed using the LDH assay. After 24h, limited toxicity was observed in all cultures (Figure 4.8). 3D cultures exhibited less toxicity than 2D, and in the multicellular model toxicity was only observed after 10 and 30 µg/ml concentrations, however large variation was observed in the multicellular spheroid model. Following 48 h incubation with AuNP, a dose-dependent increase in toxicity was observed in both 2D and 3D cultures, with greater cytotoxicity observed in 2D. In multicellular spheroids, no toxicity was observed until the 20 and 30 µg/ml concentrations, however once again much greater variation was observed in this model. It can be hypothesised that variation is due to the incorporation of a second

cell line into the model. After 72h, % cytotoxicity was similar across all culture types, with all cultures exhibiting low toxicity from 5 to 30 µg/ml. These results are well-correlated to the aforementioned studies from Chen *et al* and Zhang *et al*, among others, that suggest that 20nm AuNPs exhibited little to no induced liver toxicity in animals [368, 450, 451, 453]. Regarding AuNP induced genotoxicity, none of the concentrations tested induced any significant DNA damage in 2D or 3D cultures (Figures 4.11 and 4.14), with all % tail values being well below the acceptance criteria of 10%. Whilst it was expected that DNA damage would be reduced in spheroids when compared to monolayers, when both models were compared three out of five concentrations tested induced greater DNA damage in 3D monoculture spheroids than the 2D cultures (Figure 4.17). The fact that a 30 µg/ml dose did penetrate to the spheroid core, as observed previously in Chapter 3 (Figure 3.9), therefore interacting with more cells than the 2D model, may explain this increase in genotoxicity, however as this results is non-significant, testing a longer timepoint would be recommended to fully underpin why. When γH2AX staining of 2D HepG2 monolayers (Figures 4.18) and 3D monoculture (Figure 4.21) and multicellular spheroids (Figure 4.24) was undertaken, in a similar manner to what was observed during the comet assay, limited foci (indicative of double strand breaks) were seen across all cultures.

The second NBM assessed using the same barrage of *in vitro* pre-clinical assessment methods was LipImage™815, a novel particle encapsulating the near infrared dye IR780, a dye which in recent years has received increased attention due to its applications in in cancer photodynamic and photothermal therapy, with the goal of targeting specific cells and improving effectiveness of diagnosis [430]. Due to the main component of their composition being phospholipid bilayers (which closely mimic the mammalian cell membrane), liposomes themselves are deemed remarkably safe for pharmaceutical and medical use [454, 455]. A wide variety of conventional drugs exhibit very poor safety profiles, pharmacokinetics, limited bioavailability and high toxicity, all of which limits their use in the clinic, and to overcome these issues and to attempt to improve the therapeutic potential of these drugs, they can often be encapsulated into liposomes [456, 457]. Despite their good safety profile however, regulatory requirements mean liposomes still must be screened rigorously for their safety and efficacy profile. In the past, liposomes have been shown to exhibit hepatotoxicity, including in one study from Roursgaard *et al.* who found that cationic liposomes exhibited limited toxicity on liver cells at lower doses, however became cytotoxic after 100 µg/ml and 1000 µg/ml. Developed by REFINE collaborators at CEA-Leti, LipImage has been shown to be well tolerated in both dogs and mice [34, 392]. Accumulation of LipImage in the liver is observed, but this is more likely due to the hepatic-

biliary excretion of the lipid NBM on a whole, with the liver acting as the primary organ of metabolization and processing of lipids. The rapid uptake of liposomes in the liver, following I.V. administration, is the major cause of clinical failure and this highlighted the fact that liver accumulation and off-target side effects must be studied during the pre-clinical assessment of any potential NBMs. The liposome used in this study, LipImage™815, has been studied *in vitro* by Jacquart *et al.* in human fibroblasts, where it was shown that the IR780-loaded liposome was well tolerated and only presented as toxic at concentrations > 500 µg/ml [34]. To date no study has used hepatic monoculture or multicellular spheroids to assess toxicity of liposomes, and this is the first time LipImage has been studied using hepatic spheroids.

Evaluation of cell viability following LipImage exposure was assessed via ATP quantification. 2D monolayers exhibited the lowest viability, with the highest concentration, 500 µg/ml, yielding $82.68\% \pm 12.10$ after 72h (Figure 4.5 A). When compared to HepG2 monolayers, HepG2 monoculture and multicellular spheroids exhibited a greater % viability, with $93.50\% \pm 22.81$ and $90.59\% \pm 2.64$ viability observed following a 72h incubation of 500 µg/ml LipImage in both models (Figure 4.5 B and C).

Whilst non-significant (Table 4.3), it can be hypothesized that the increased viability in the spheroid models is due once again to the difference in exposures, with flat 2D monolayers being subject to greater interaction with LipImage than the spheroid cultures. The ability of LipImage to induce cytotoxicity was then assessed via LDH assay. After 24h incubation with LipImage no toxicity was observed in 2D cultures until 500 µg/ml treatment in 2D monolayers or multicellular spheroids ($1.69\% \pm 1.23$ and $2.53\% \pm 6.51$ respectively) (Figures 4.8 A and B). Following 48h, no toxicity was observed in 2D cultures or 3D monoculture spheroids until 500 µg/ml treatment. Each concentration induced low levels of toxicity in multicellular spheroids (Figure 4.9 C), with 500 µg/ml inducing $2.83\% \pm 0.64$. The largest cytotoxicity was observed in the 2D model after 72h exposure ($10.83\% \pm 3.59$ for the 500 µg/ml treatment). These results are in agreement with cytotoxicity assessment undertaken by Jacquart *et al.* who found that LipImage was well tolerated *in vitro*, exhibiting no significant toxicity until doses above 750 µg/ml [34]. When assessing for its ability to induce DNA damage, LipImage unsurprisingly exhibited little genotoxicity in 2D monolayers and 3D monoculture spheroids, with % tail values for all concentrations less than the 10% QC/QA acceptance criteria (Figures 4.12 and 4.15). Slightly more DNA damage was observed in the 3D spheroids, however this was non-significant. These results, as unloaded liposomes have been shown to exhibit no significant genotoxicity *in vivo* in the liver, even in the repeated dose study where liposomes were administered three times a week

[458]. The lack of DNA damage seen using the comet assay was further validated from the γ H2AX staining. As observed in Figure 4.22, no foci were present following 24h treatment with 500 μ g/ml LipImage, and few formed in HepG2/LX-2 multicellular spheroids (Figure 4.25).

Finally, as there is limited literature available on this potentially clinically relevant material, the data and results achieved in this study is of importance to the potential clinical development of LipImage as medical probes for advanced imaging. The final NBM used in this study was NR668-loaded PEBCA (2-ethyl-butyl cyanoacrylate), a subset of the PACA family of NPs, supplied by collaborators at SINTEF [409]. Despite the expectation that polymeric NPs are biodegradable, careful acute, sub chronic and chronic toxicity studies targeting hepatotoxicity is essential, as some polymeric NBMs have been shown to induce hepatotoxicity, including PLGA which has been shown to reduce ALT and AST activity [459], with hepatotoxicity also noted following histology and H&E staining [429]. To date, the PACA subset of polymeric NPs are being used in multiple late-stage clinical trials [460-462]. Despite numerous studies detailing the cytotoxicity of PACA NPs, these studies generally lack consistency and only evaluate one specific subset of the PACA family and not considering other formulations (i.e., PEBCA v. POCA v. PBCA etc.). The most comprehensive study of PACA NBMs has been undertaken by Sulheim *et al.* who assessed 19 varying PACA NBMs with sizes varying from 100 – 200 nm (including the one used in this study, PEBCA) in 12 differing cell lines using the LDH and MTT assays. Here it was found that the IC₅₀ value for PEBCA was 68 μ g/ml, a value higher than that reported by Lira *et al.* [463] but lower compared to results reported by Kolter *et al* [464].

The toxicity assessment of neither PACA NPs on a whole, or the chosen PACA NBM for this study (NR668 loaded PEBCA) has been undertaken previously in hepatic monocellular or multicellular spheroids. No *in vivo* study to date has assessed the specific PACA NBM used in this thesis, however a PK animal study due to take place in January 2021 will greatly add to this work, providing more information about the fate of this NBM in the body. Many animal studies have been undertaken however using polymeric NBMs in general. Polymeric NBMs are found to be well tolerated *in vivo*, with no histological differences observed in liver tissues in many studies [370, 465]. ATP quantification revealed a lower percentage viability in 2D cultures, i.e. 79.76% $\pm$ 3.64, 79.61% $\pm$ 15.36 and 78.07% $\pm$ 11.54 following 24, 48 and 72h incubations with 30 μ g/ml PACA (Figure 4.6 A), compared to 3D monoculture and multicellular spheroids. This result is comparable to results obtained from Sulheim *et al.* who assessed PEBCA toxicity using the same assay and cell type as in this thesis (Supplementary Figure S4.3). HepG2 monoculture spheroids exhibited viability of

91.66% $\pm$ 10.24, 84.91% $\pm$ 3.59 and 81.47% $\pm$ 6.80 after 24, 48 and 72h treatments with 30 μ g/ml PACA (Figure 4.6 B). In a similar manner, multicellular spheroids also exhibited greater viability compared to 2D monolayers, with % viabilities of 95.91% $\pm$ 2.38, 90.56% $\pm$ 2.72 and 87.68% $\pm$ 14.16 for the same exposure (Figure 4.6 C). Multicellular spheroids also presented as more viable than monoculture spheroids. It can be hypothesised that this is due to the reduced uptake of PACA in multicellular spheroids compared to their monoculture counterparts, as observed previously in Chapter 3 (Figure 3.13). In comparison to all the NBMs assessed in this study, PACA exhibited the greatest cytotoxicity across all concentrations for the 2D model (Figure 4.10 A) After 24h, % cytotoxicity was low in both 3D monoculture spheroids and multicellular spheroids, with the HepG2/LX-2 model exhibiting no toxicity at all after this time (Figure 4.10 B and C). After 48h, a dose-dependent increase was observed in the 2D monocultures and 3D HepG2 spheroids in comparison to the multicellular model with exhibited low non dose-dependent toxicity across all concentrations. Results obtained in the 2D model for 24h were similar to those published from Sulheim *et al.* [409], who used the same assay and cell type (Supplementary Figure S4.3), and 24 and 48h exposures with PACA also appear to be similar to preliminary results obtained during an interlaboratory comparison within the REFINE project by collaborators at SINTEF in their study of the same NBM using the same cell line and assays (data to be presented at a later date).

The most stark differences within my results between the cultures is observed after 72h. After 72h, significant cytotoxicity was observed in the 2D model, with the highest concentration, 30 μ g/ml, exhibiting almost half the cytotoxicity of the Valinomycin positive control, i.e. 20.87% $\pm$ 3.43. (Figure 4.13). A dose-dependent increase in cytotoxicity was observed in the 3D model, however values were significantly less than in 2D, with the highest concentration exhibiting 6.53% $\pm$ 3.40 cytotoxicity. The multicellular 3D spheroid model exhibited toxicity at all concentrations, however for the 20 and 30 μ g/ml concentration toxicity was lower than the other models (i.e. 3.57% $\pm$ 1.04 after 30 μ g/ml. When assessed for their ability to induce DNA damage, PEBCA NBMs appeared non-genotoxic, with all concentrations and timepoints assessed exhibiting no % tail values above the acceptance criteria in any of the models (Figure 4.13 and 4.16).

As stated above, this work is novel in the sense that has been developed with the developer of the PACA NBM, and thus the data set generated is original and important as studies on cytotoxicity and genotoxicity of this material to date are scarce. The lack of genotoxicity observed in the comet assay was further validated by the lack of foci seen from γ H2AX staining (Figures 4.23 and 4.26).

In this study, exposure of HepG2 2D and 3D models did not induce any significant cytotoxic or genotoxic effects, with the exception of a 72h treatment of PACA, inducing significant cytotoxicity in 2D monolayers. This is unsurprising considered various studies have reported that PEGylated AuNPs, liposomes and polymeric based NBMs are less toxic than other NBMs, i.e. metal oxides [68, 453, 466, 467]. Whilst none of the NBMs screened induced significant enough toxicity to fully underpin the differences between each of the models, some small differences in sensitivity between the 2D monolayer cultures and 3D monoculture and multicellular spheroid models were observed across all experiments. Various studies have shown the differences in sensitivity to toxicity induced in both 2D and 3D cell culture models, with 3D models appearing more resistant to toxicity of various chemicals or drug compounds. From viability and cytotoxicity assessment it appeared that 3D spheroids overall are more resistant to the effects of NBMs than 2D monolayers (this is noted especially following PACA exposure). It is possible that the more tissue-like morphology and functional relevance of hepatic spheroids is the reason for this [223]. It is also possible that this is due to the exposure scenarios presented in each culture. Whilst cells in two dimensions grow in monolayers on the bottom of flat wells, spheroids are more suspended, resting above the bottom of U-shaped wells. Each culture will only be exposed to the same NBM concentration provided that the exposure is a stable colloidal dispersion throughout the experimental timepoints, which would be the case for some metallic NBMs (e.g. ZnO and TiO₂). It is possible that aggregation and sedimentation of the NBMs used in this study would reduce the NBM load in spheroids, whilst increasing the concentration of NBMs reaching the cells of the 2D monolayers. This may go some way to explaining the increased toxicity and reduced viability of the 2D cultures compared to the spheroids. As spheroid cultures are exposed to NBMs only on the spheroid surface, the exposure of cells is highly dependent on the ability of the NBM to penetrate inside the spheroid [284]. Method of exposure, i.e. whether NBMs are exposed statically or dynamically also plays a role. Cell death inside spheroids could occur via certain signalling pathways activated via the cells on the spheroid surface. Elje *et al.* have shown that short exposures of spheroids to the genotoxic compound H₂O₂ was not sufficient enough to induce similar amounts of strand breaks between 2D and 3D HepG2 cultures, with damage approximately ten times higher in the 2D model. It can be hypothesised that the short exposure time, i.e. 24h, the longest timepoint used in the genotoxicity study in this thesis, was too short for the compounds to reach the spheroid interior and induce genotoxicity across all time points and NBMs in this study [468]. However, in this thesis, it was shown that 3D cultures did exhibit greater % DNA damage when compared to the 2D cultures across various concentrations for each NBM

tested. This is in agreement with the work from Elje *et al.*, who found that DNA damage induced by MMS in HepG2 spheroids was greater than 2D monolayers [469] and interestingly, a study from Shah *et al.* who also found that 3D HepG2 spheroids were more sensitivity to genotoxicity than 2D cultures [470]. This increased sensitivity for DNA damage was also observed in a study from Mandon *et al.* who demonstrated enhanced DNA damage in a HepaRG spheroid models following exposure to 11 various chemicals [471]. Whilst it was shown in this study (Chapter 3) that the chosen NBMs could successfully reach the spheroid core, this was only observed at the highest concentration for each material, and not at the lower concentrations tested in the comet assay (Chapter 3 Figure 3.9, 3.10 and 3.11). A study by Fledderman *et al.* have shown that SiO₂ particles were distributed throughout whole HepG2 spheroids when they were mixed with cell suspensions prior to seeding [283]. It may be worthwhile to apply this treatment method to extend this study.

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, cytotoxicity assessment of NBMs, in 2D cultures, 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. In this chapter, 3D monocellular and multicellular spheroid models were assessed for their ability to be more predictive of *in vivo* hepatotoxicity than conventional 2D cultures. The data presented in this chapter correlates well to results obtained in *in vivo* studies of the similar NBMs, or the same where available. This shows great potential for the use of HepG2 spheroids as an intermediate stage in pre-clinical testing, bridging both 2D *in vitro* experimentation and *in vivo* animal models, and thereby extending the current cellular level cytotoxicity assessment to the tissue level. This, in turn, greatly improves the predictive power of *in vitro* experimentation in the assessment of NBMs. This is also a critical consideration with regard to the concept of the 3Rs - replacement, reduction and refinement—where the goal is to reduce the numbers of animals used in *in vivo* experimentation, by developing robust, accurate and *in vivo*-like *in vitro* pre-clinical assessment models.

With this being said, the development of new experimental model is not exempted from limitations. For instance, greater variation in assay results was observed in the spheroid models, which made it difficult to achieve statistically significant results. There is also room for modifications to experimental protocols, and cell numbers and NBM concentrations must

be highly controlled in order to compare as accurately as possible the responses in both cultures. All these have been attempted to be kept consistent during the SOP development and the inter laboratory study.

4.5 Conclusion

- Before undertaking the work involved in this chapter, it could be predicted that HepG2 spheroids would act as better predictors of NBM-induced cytotoxicity and genotoxicity than 2D monolayers, due to their more *in vivo*-like morphology, increased functionality and increased sensitivity to hepatotoxins.
- Hepatic spheroids appear more resistant to the cytotoxic effects of NBMs compared to 2D monolayers. It can be hypothesised that this is due to the more tissue-like morphology and *in vivo* relevance and is in accordance to what is noted in literature.
- Spheroid models appear more sensitive to the genotoxic effects of NBMs, however variation is observed.
- Finally, it can be concluded that the alkaline comet assay can be successfully be applied to the pre-clinical assessment of NBM-induced genotoxicity, using the adapted SOP presented in this chapter. It can further be validated using γ H2AX staining.

Chapter 5 – Overall conclusions and future work

5.1 Chapter summary

As stated at in Chapter 1 of this thesis, the overall aim of my Ph.D. project was to determine if a 3D hepatic spheroid model could be a better predictor of nanobiomaterial (NBM) safety than traditional 2D cell cultures, and if they could be successfully implemented into the pre-clinical assessment cascade of NBMs. The development of a more *in vivo*-like human relevant hepatic model may potentially be a better predictor of NBM safety than what is currently available , ultimately overcoming some of the hurdles currently associated with NBM translation, as well as potentially bridging the gap between existing *in vitro* screening methodologies and *in vivo* animal models, in accordance with the 3Rs concept of replacement, reduction and refinement, for assessing the toxicity response of these materials. To date, the use of 3D models, specifically of the liver, is very limited in nanomedicine research, making the work presented in this thesis a significant step forward for our field. This research may also contribute at reducing the costs associated with and speed up the process of NBM pre-clinical assessment, producing safer NBM-based medical products and consequently minimizing the number of adverse effects seen in patients, by preventing NBMs and materials with potentially hepatotoxic liabilities reaching clinical trials. This concluding chapter will discuss the main conclusions of my Ph.D. research project, and the ongoing efforts to overcome the issues seen regarding the translation of NBM products from bench to bedside.

5.2 Overall conclusions

As presented in Chapter 1, from the scientific literature it has emerged that oversimplified models such as 2D monoculture cellular models are unable to detect up to 40% of hepatotoxic materials [472]. Therefore, there is an unmet need for more advanced and *in vivo*-like techniques to be adopted within a regulatory science framework. Details of the most common systems for assessing liver toxicity are reported in Figure 5.1.

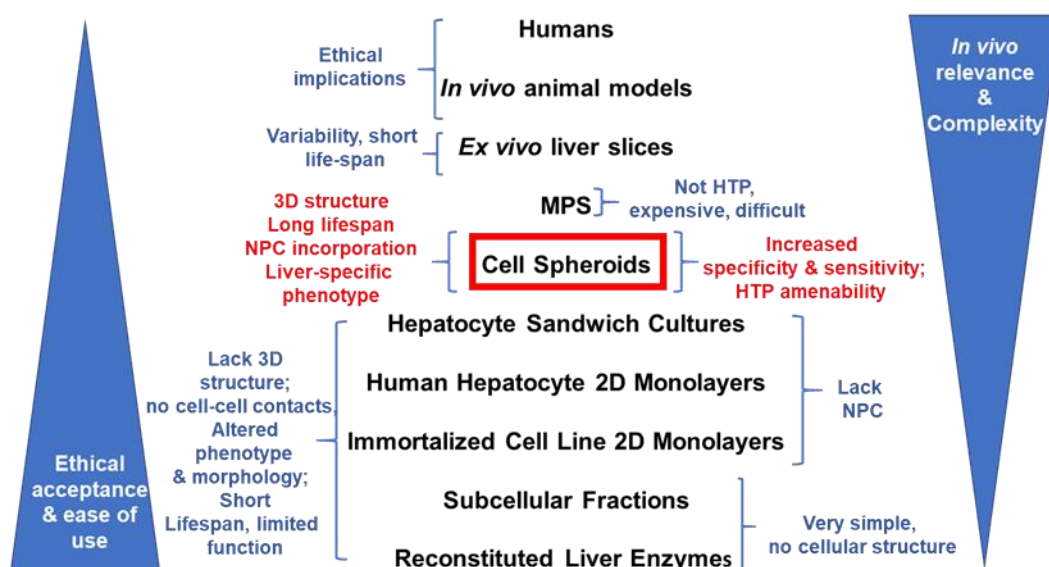


Figure 5.1. Common liver models; advantages and disadvantages.

A wide variety of liver models are currently available for assessing drug and NBM toxicity, ranging from ones which are the most complex and closely resemble humans (i.e., actual human trials and animal models), to others which are more ethically acceptable and easy to use, but greatly reductionist in nature. Sitting between each of these models are cellular spheroid models. Spheroids have a longer lifespan, a more liver-specific phenotype, increased sensitivity and specificity compared to 2D cultures, and are also amenable to high throughput (HTP) screening, whilst also being ethically acceptable and easy to manipulate. Due to their advantages over other reductionist in vitro models, spheroid models have now been optimised and validated for use in material safety screening.

Advanced *in vitro* models of the liver are very much needed in order to investigate and detect human hepatotoxicity prior to undertaken human clinical trials, and in recent years, some effort has been put by the nanomedicine and nano safety community at investigating how 3D models can be accepted for drug and NBM screening at regulatory level. Today, both reductionist 2D *in vitro* models and *in vivo* animal models often fail at detecting NBM-induced hepatotoxicity, due to issues ranging from the inability of 2D cell culture to mimic the natural structures found in human tissues like the liver, the non-linear dose-toxicity relationships the exhibit and their inability to predict downstream or chronic effects, to the fitness of animals to adequately predict human responses due to interspecies variation. A bulk of evidence is available in literature which shows that 3D hepatic cell culture models may overcome these critical issues. These 3D models display more *in vivo*-like structure and

function when compared to 2D monolayer cultures, in turn yielding an increased sensitivity to hepatotoxins [84, 105, 233, 299, 335]. Due to their relative ease of culture compared to other more complex methods, their reproducibility, and amenability to high throughput (HTP) assays, one of the most popular *in vitro* 3D models which has emerged to potentially bridge the gap between reductionist models and *in vivo* animal models, and the model of choice for this thesis, are hepatic spheroids. Today, pharmaceutical companies such as AstraZeneca and commercial companies like Mimetas routinely use liver spheroids in their testing regimes and for assessing mechanistic hepatotoxicity [473, 474]. When discussing the future of advanced *in vitro* models, it would also be remiss to not mention their implications for *in vivo* animal studies. In recent decades, the potential of replacing animal models with more human-relevant *in vitro* 3D models or incorporating 3D models into the pre-clinical assessment cascade as a bridge between the two conventional screening models, has been widely discussed. This is vitally important in order to improve on the low 50% detection rate for human hepatotoxins currently observed in animal studies [475]. Due to the unethical nature of using and causing harm to animals in research, an area of constant criticism in the development process for drugs and NBMs alike is *in vivo* animal experimentation. With the well-documented variations in genetic differences between species, coupled with environmental differences, huge questions arise over whether animal models are relevant to toxicity at all [476]. More complex, human-relevant *in vitro* pre-screening models have great potential for assessing ADME, efficacy and side effects of drugs and NBMs, with each of will help determine efficacious and safe doses for both in human clinical trials [261, 477-479]. With this being said, despite the fact that it is unlikely that animal studies will be fully abolished, there is the potential for the alternative and more human-relevant *in vitro* testing systems discussed in this thesis and above to potentially replace some of the many animal-based assays used today. In the future, these specialised liver models may play a vital role in the drug development process.

With the importance of these new, human relevant *in vitro* models in mind, Chapter 2 dealt with the development and validation of 3D HepG2 hepatic mono- and HepG2/LX-2 multi-cellular hepatic spheroid models. These models were successfully developed using these cell types cultured using the scaffold-free method of non-adherent 96-well plates, with various liver specific parameters assessed, including some not previously investigated by other works. Firstly, two scaffold-free methods for culturing 3D spheroids were assessed, the InSphero™ hanging drop system and CellStar® cell repellent plates, with it being determined that the cell repellent plates were the optimal method to carry forward for all future experiments. The work presented is the first time, to the best of my knowledge, that HepG2

spheroids have been cultured in these specific plates, adding to the knowledge which previously exists for non-adherent environments. Spheroid preparation technique was optimized, and the liver-specific biology of HepG2 spheroids was validated, with key parameters assessed including lifespan, viability, cell count, 3D morphology, and formation of secondary structures. From this work it was found that HepG2 spheroids bared a strong likeness to *in vivo* tissue. In addition to this, from functional analysis, i.e. albumin production and MRP2 transporter expression and functionality, it was observed that HepG2 spheroids exhibited an increased likeness to the *in vivo* environment in terms of functionality, in accordance to what is observed in literature. HepG2 spheroids were also found to be more sensitive to panel of hepatotoxins, namely fialuridine, diclofenac, trovafloxacin and acetaminophen, (each of which was chosen due to their varying mechanisms of toxicity with spheroids displaying greater sensitivity to these drugs their 2D monolayer counterparts. Furthermore, sensitivity to trovafloxacin and diclofenac observed in 3D spheroids was comparable to what is noted in literature for primary human hepatocytes. This evidence confirms that HepG2 spheroids have the potential to detect hepatotoxins with greater sensitivity than 2D culture models. The effect of including a non-parenchymal cell (NPC) line was also assessed, with the chosen cell line, hepatic stellate cells (LX-2s), further increased the sensitivity of the model to hepatotoxins. As the spheroids were found to exhibit greater sensitivity to hepatotoxins, it could be hypothesised that they would also have the potential detect NBM toxicity more sensitively. This work is a significant advance as to date only two publications have incorporated LX-2 cells with hepatocytes in a liver spheroid model, with neither of them using said model to assess NBMs.

Following on from Chapter 2, the exposure response to three, purpose-specific representative NBMs which can be applied to medical technology, namely a dye-loaded diagnostic liposome (LipImage), a far red-loaded polymeric NP (PACA), representative of a drug delivery carrier, and a benchmark metallic NP, was qualitatively assessed in Chapter 3, in order to determine the biological relevance of the models developed. The work presented in this chapter is the first time that the NBMs LipImage and PACA were assessed using 3D liver spheroids (these materials were obtained through collaboration in the REFINE project, with PACA specifically designed and synthesized by collaborators at SINTEF, Norway as part of this project). The work presented is also the first time a commercial 20nm PEGylated AuNP (NanoComposix) was assessed in liver spheroids. This work was carried out in both 2D monocultures and 3D spheroids (mono- and multi-cellular) with results compared, when possible, to *in vivo* animal studies of the same particles, in order to determine the biological relevance of the spheroid models. Results in these chapters showed that each of the chosen

NBMs successfully accumulate with spheroids in a concentration dependent manner and are localized to the cytoplasm. Less pronounced distribution of NBMs was observed in the multicellular spheroids when compared to monoculture spheroids. Within the REFINE project, this work was only undertaken by TCD.

In the final results chapter (Chapter 4), the relevance of the spheroid models to assess NBM-induced hepatotoxicity and genotoxicity was studied. In this chapter, quantitative assessment and comparison of cell viability, cytotoxicity and genotoxicity of 2D monocultures and 3D monoculture and multicellular spheroids when incubated with the three representative NBMs, were assessed using a variety of techniques. It is important to point out that once again this work was carried within the REFINE project, a large European Horizon 2020 project (grant agreement ID: 761104) which focuses on developing regulatory science frameworks for NBMs, with the interaction and scientific exchanges I had with expert partners in toxicology and safety assessment of nanomaterials being very beneficial to my work. Protocols and assays which could be successfully applied for the assessment of NBM toxicity were assessed. With regards to the aspect of hepato- and geno-toxicity existing OECD guidelines were adopted and revised for the assessment of NBMs via 2D and developed 3D biological models. To contribute to further expand the availability of models for safety testing, a large effort was invested at TCD to lead and coordinate a large-scale genotoxicity study. My work contributed to the developed SOP which was focused on the screening of NBMs. This SOP was robustly tested under a three partner interlaboratory comparison study. In Chapter 4 (Section 4.3.1 and 4.3.2), from the hepatotoxicity assessment using viability and cytotoxicity assays, it was determined that hepatic spheroids are more resistant to the toxic effects of NBMs than conventional 2D monolayers cultures. It can be hypothesised that this is due to their greater tissue-like morphology and greater *in vivo* relevance, previously detailed in Chapter 2. Regarding genotoxicity, spheroid models were found to respond more sensitively to NBM-induced DNA damage, however non-significantly (Chapter 4, Section 4.3.4.3). From these results it can be determined that hepatic spheroids can be successfully used to bridge the gap between existing *in vitro* screening methodologies and *in vivo* animal models, for assessing the safety profiles of novel NBMs that can be used in medical technology. This work is the first time that the cytotoxicity of these specific NBMs have been assessed using spheroids, specifically hepatic spheroids, and the first time they have been assessed for their genotoxic potentials, using an SOP specifically developed as part of my project.

Today, there is a pressing need for the development of both robust and industrially acceptable methodologies for assessing the acute and chronic hepatotoxicity of NBMs. The

work presented in this thesis significantly contributes to advancements in the use of hepatic spheroid models in the pre-clinical assessment of NBMs, an area which is still in its early stages and an area where there are still no validated SOPs or workflows for doing so at present. Building on experimental evidence presented in the first two results chapters of this thesis, it was shown in Chapter 4 that the hepatic spheroids developed for this study could be successfully applied to the pre-screening of NBMs and used as suitable predictors of their hepatotoxicity. By undertaking this work and through the adaptations of pre-existing OECD guidelines for assessing NBM-induced hepatotoxicity, a significant knowledge gap is filled. In the coming months, across an interlaboratory comparison for assessing NBM hepatotoxicity using liver spheroids within the REFINE project, the results and workflows presented in this thesis will be further validated through the standardisation and robust testing of the new SOPs based on the protocols and assays for 3D biological models used in my work, in a similar manner to the already validated and accepted genotoxicity SOP presented in Chapter 4. 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) [286], Elje *et al.* (2020) [284, 469] and Fleddermann *et al.* (2019) 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 thesis. To date, very few publications have used 3D liver models for assessing distribution and toxicity of NBMs. One key study worth mentioning from Chelobanov *et al.* (2020) [445], has assessed the uptake of various forms of AuNPs in HepG2 spheroids using TEM, however this study relatively isolated, and to the best of my knowledge the work in this thesis is the only time hepatic spheroids have been applied to both the assessment of distribution of the NBMs used, and their *in vitro* toxicity screening, namely hepatotoxicity and genotoxicity, making the work presented in this thesis a significant advance in the field. The protocols put forward in this thesis and the validated SOP for assessing NBM-induced genotoxicity will also no doubt assist widely in future studies of NBM hepatotoxicity using 3D liver models. With all this being said, the HepG2 3D spheroids presented in this thesis can be described as a novel, 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 one month, 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 in this thesis is a positive contribution to the development of advanced *in vitro* models.

5.3 Future work

There are many aspects of my work which will be expanded further

Firstly, there is the potential that the characterization of the established models be expanded to other secondary endpoints, to provide a better description of the *in vitro* model as a whole. In this thesis, the key hepatic transporter most often cited in studies of liver spheroid models, i.e. MRP2, was assessed. In terms of model characterization, analysis of the expression of hepatic uptake and export transporter other than MRP2 may be of value. In order to further validate the presence of bile canaliculi it may be useful to probe the localisation and functionality of the Pgp transporter. The Pgp transporter, in a similar manner to MRP2, is an ATP powered efflux pump which plays a role in the transport of hundreds of compounds including therapeutic drugs. Other apical and basolateral membrane transporters, such as MRP1, SLC28A3 and SLCO3A1 (apical) and BSEP (basolateral) may also be assessed.

To further complement the functional analysis of spheroids, synthesis and secretion of urea may be studied by using the same methodology used for the human serum albumin (ELISA). Furthermore, as one of the primary functions of the human liver is homeostasis of various substances and molecules, which not only includes metabolism and degradation of compounds, but also synthesis of substances and detoxification of xenobiotics, it would be useful to assess activity of various CYP enzymes [85]. A vast amount of the metabolic processes seen in the liver are performed by different Phase I CYP enzymes, whose functionality is critical for efficient liver function [480]. Therefore, the expression and activity of CYP enzymes is a useful indicator of liver-specific functionality in spheroids. CYP enzymes including CYP3A4 and CYP2E1, crucial CYP enzymes responsible for metabolism of xenobiotics including diclofenac and acetaminophen, have been previously shown to be expressed in hepatic spheroids when compared to 2D hepatic cultures in many studies [481]. Analysing a full panel of not only Phase I but also Phase II enzymes, such as UGT, GST and NAT, would be a useful addition to the barrage of experiments presented to fully determine metabolic capacity of the hepatic spheroid model.

Many therapeutic compounds can cause liver injury in manners often unrelated to the therapeutic mechanism of action. The mechanism of toxicity of hepatotoxic drugs varies from compound to compound and the exact mechanism by which a drug causes injury being

often disputed or unconfirmed. Some drugs have been shown to cause toxicity by the formation of a reactive metabolite, such as paracetamol (acetaminophen), with it being suggested that other compounds such as fialuridine and diclofenac cause toxicity due to their impact on mitochondria. By using Seahorse technology and the mitochondria stress test assay, it will be possible to assess mitochondrial activity in hepatic spheroids and underpin the effects of both drug compounds and NBMs. This work may be undertaken in collaboration with the TCD Department of Surgery. A major goal of the work presented is its expansion to a more tissue-mimetic model. If issues regarding availability and reproducibility can be overcome, the incorporation of NPCs such as Kupffer cells into the model would be of great benefit in expanding this study. In my Ph.D. project I developed a multicellular hepatic spheroid model combining HepG2 cells (hepatocytes) with LX-2 hepatic stellate cells (HSCs). Various studies have shown that *in vivo* NBMs interact with the Kupffer cells first, followed by liver sinusoidal endothelial cells (LSECs), before reaching HSCs. Approximately only 7% of NBMs are found in hepatocytes following administration [395]. However, due to difficulties in cell sourcing of Kupffer cells and other cells such as LSECs, the integration of HSCs and LX-2 was preferred within my Ph.D. project for the purpose of establishing a protocol for the proof-of-principle creation of multicellular spheroids.

In Chapter 4, the hepatic spheroids developed in this study were applied to the pre-clinical assessment of the chosen NBMs, with common endpoints analysed, namely viability, cytotoxicity and genotoxicity, and area often overlooked in NBM pre-screening. None of the NBMs however exhibited toxicity in the *in vitro* assays significant enough to make a full determination on whether 2D or 3D models were a better predictor of toxicity. It would be of great benefit to this study to incorporate into this study an NBM which causes significant cell death in order to fully compare the responses of mono- and multi-cellular spheroids and to validate the model. It would be useful to extend this study using Doxil[®], liposomal doxorubicin, and its non-liposomal free-drug form doxorubicin. As a significant amount of literature is available on the *in vitro* toxicity of doxorubicin and liposomal doxorubicin in liver cells [482-485], in combination with the numerous *in vivo* animal studies and human trials which have been undertaken using Doxil[™] [486-491], undertaking quantitative assessment of these materials and incorporating them as a final step would be of great benefit in this study and would act as a means of alpha testing the models presented. As toxicity will be observed following treatment with Doxil[™], it will also be possible to define the predictive value of the model and calculate specificity and sensitivity. To the best of my knowledge, no such study has been undertaken previously, i.e. the pre-clinical screening of 3 NBMs and

a benchmark chemotherapeutic, in either 2D hepatic monolayers or 3D monoculture and multicellular hepatic spheroids. This fact, coupled with the *in vivo* animal biodistribution studies using the AuNP and NR668 PEBCA NBMs described in this thesis which are due to be undertaken in early 2021, will work towards fully validating the spheroid models presented in this thesis.

In recent years *in vitro* models of the liver have become more and more advanced, incorporating new state of the art techniques. As multicompartmental microfluidics devices are now commercially available for many sources, it is not possible to have a sustained flow of culture media from one cell to another, therefore replicating the interactions between all cell types in varying organs and tissues in the human body. By using a microfluidics or organ-on-chip (OOC) models, it is possible to incorporate various NPCs with hepatocytes, allowing the formation of a full organ system in one model. The development of OOC devices was brought about due to a pre-existing need for more representative *in vivo* testing tools, which could overcome the glaring discrepancies seen with pre-existing screening methodologies and which may, in the future, render animal testing obsolete. Multi-organ systems (MOCs) or body-on-a-chip models may also be of use, incorporating cells from various other relevant organs, i.e., kidney, GIT. To date, a small number of organ or body-on-chip simulations have proven useful, including work from Tostoes *et al.* and Lee *et al.* [233, 275].

Literature is scarce on combining spheroids and microfluidics for assessing NBMs, however to date a small number of studies have attempted to combine spheroids and microfluidics for assessing NBMs, with studies from Albanese *et al.* [492], and Chen *et al.* [493] having attempted to use tumour-on-chip models in an attempt to underpin NP uptake and transport. Esch *et al.* have combined two *in vitro* models, a human intestinal epithelium model of Caco-2 and mucin producing TH29-MTX cells, and HepG2/C3A cells in one microfluidic device. By using this device, the compounding effects of 50nm polystyrene NPs could be assessed, with liver injury detected at lower NP concentrations than expected from single tissue experimentation [494].

To extend the work presented in this thesis, a goal is to incorporate parenchymal (hepatocytes) and non-parenchymal (Kupffer) liver cells, with an endothelial cell line that has angiogenic potential in a commercially purchased microfluidic chip device (the OrganoPlate LiverTox system using the OrganoPlate 2-lane chip) in a similar manner to work undertaken by Kirsten Birscak and colleagues at Mimetas [495]. With the huge advances made in stem cell technology over the last ten years, endothelial cells derived from induced pluripotent stem cells (iPSCs) would be hugely beneficial to the model, allowing a

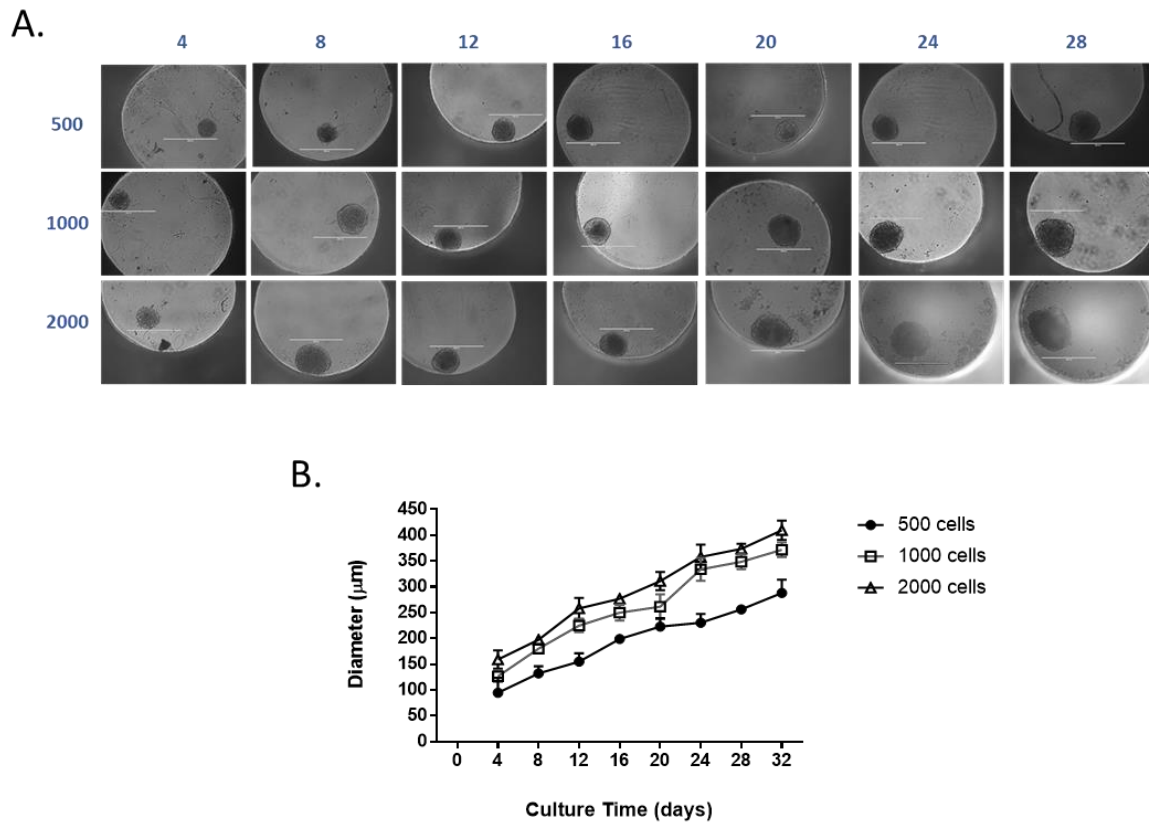
promising alternative and renewable source of cells when compared to traditional endothelial cells. Provided the practical difficulties and challenges such as issues with shear stress removing cells or NBMs from the system, as well as issues with standardization and scale-up can be overcome, these models would be of huge benefit to the study both the acute and chronic toxicity of the chosen NBMs.

Chapter 6

Supplementary

6. Supplementary

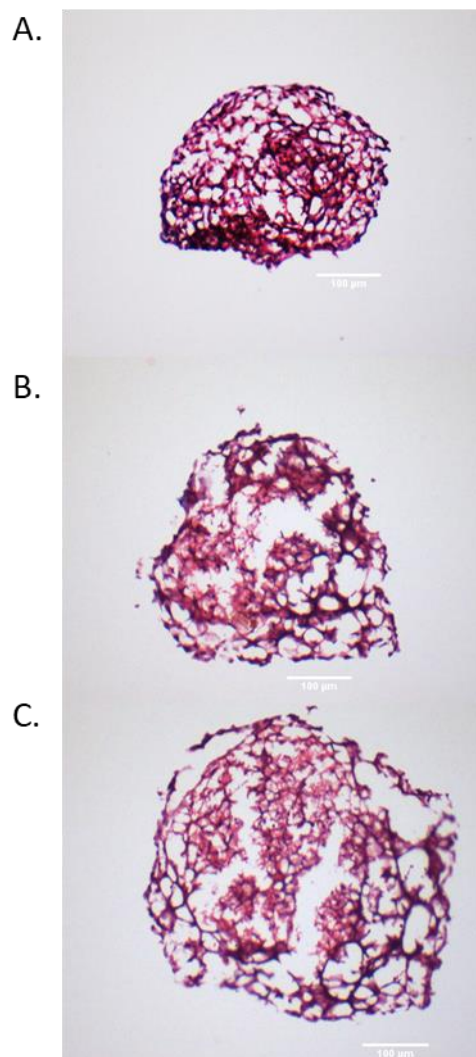
Supplementary Figure S2.1.



Supplementary Figure S2.1. HepG2 spheroids formed using InSphero™ hanging drop system.

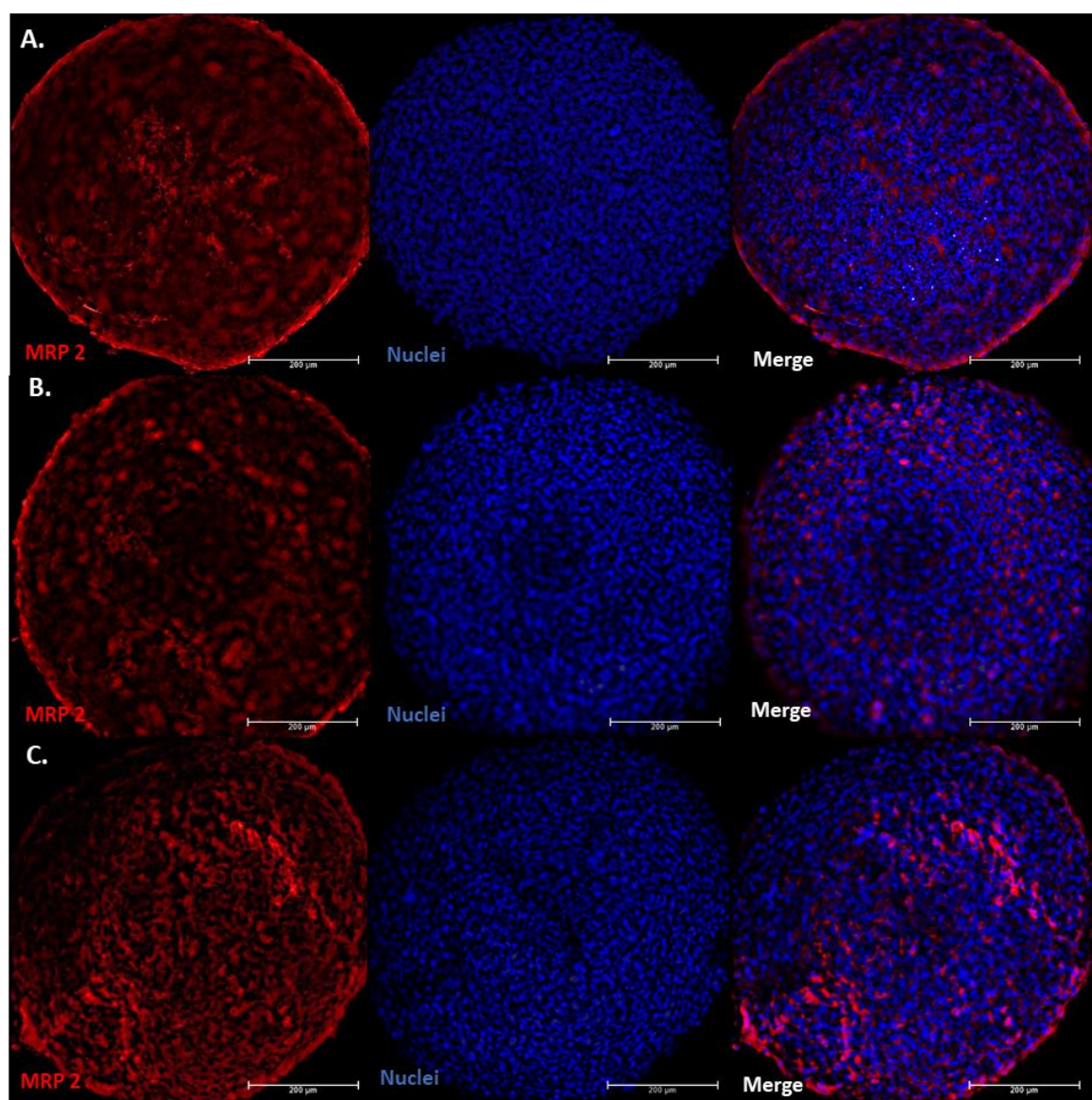
EVOS™ images of spheroids cultured from 500, 1000 and 2000 cells in 100 μ l culture medium using InSphero™ hanging drop system over 28 days. Scale bar = 400 μ m. Growth curve of spheroids cultured using InSphero™ hanging drop plates. Spheroid diameter (μ m) was plotted against culture time (days). Data are represented as mean $\pm$ SEM (n= 6 replicates).

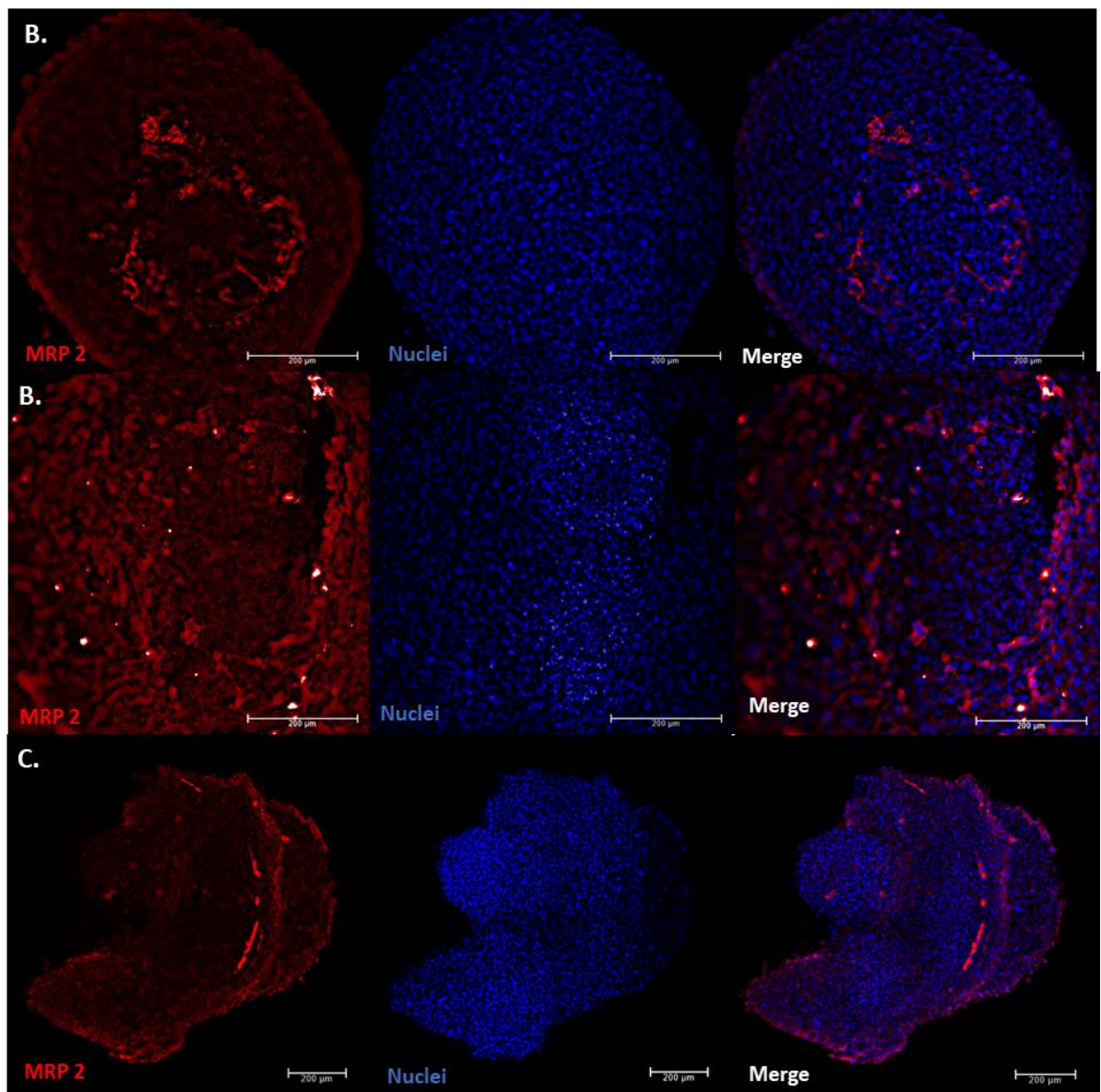
Supplementary Figure S2.2.



Supplementary Figure S2.2. Cryopreservation of HepG2 spheroids. For histological analysis, cryopreservation using OCT medium was trailed prior to paraffin embedding. Cryopreservation was found to damage structure of spheroids greatly, therefore paraffin embedding was carried forward for all further experiments. (A) 1000 cells, (B) 5000 cells and (C) 10000 cells in 100 μ l culture medium. Scale bar = 100 μ m.

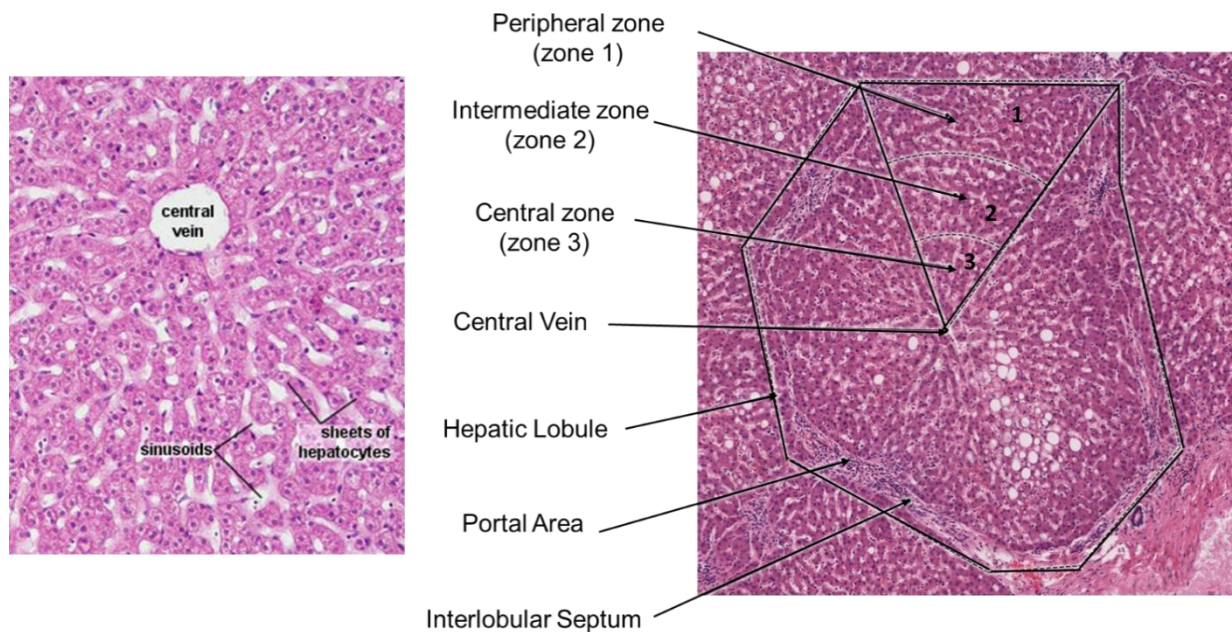
Supplementary Figure S2.3.



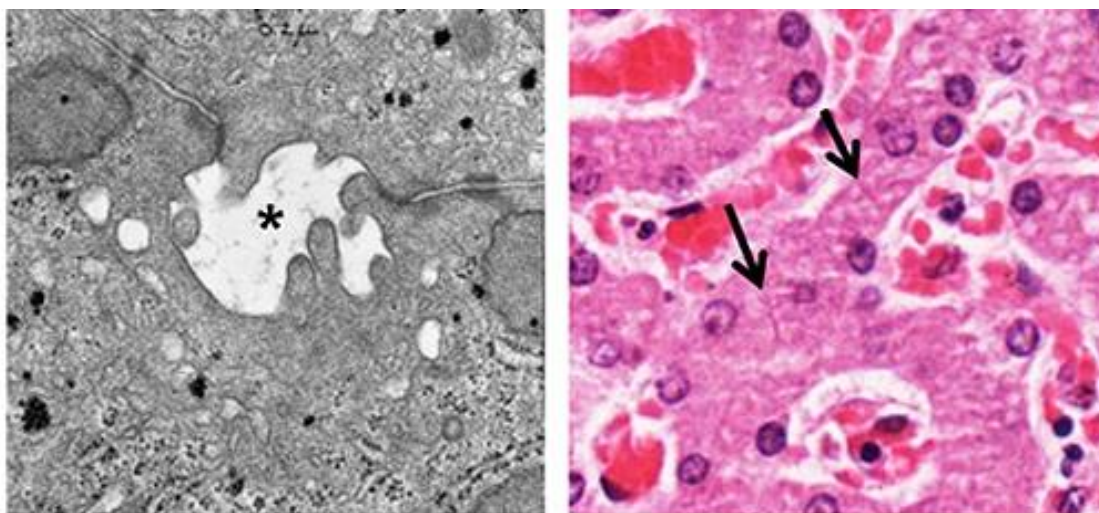


Supplementary Figure S2.3. Localisation of MRP2 transporter in HepG2 spheroids. HepG2 spheroids were formed from (1) 5000 and (2) 10000 cells and cultured for 4, 11 and 18 d. Immunofluorescent staining was undertaken with MRP2 antibody (red – canalicular transporter) and Hoechst (blue - nuclei). Quantitative LSCM imaging was undertaken with the assistance of Dr Gavin McManus, Trinity Biomedical Sciences Institute, Trinity College Dublin, using a Leica system and 20 x magnification. Image processing undertaken using LAS X software. Scale bars = 200 μm.

Supplementary Figure S2.4.



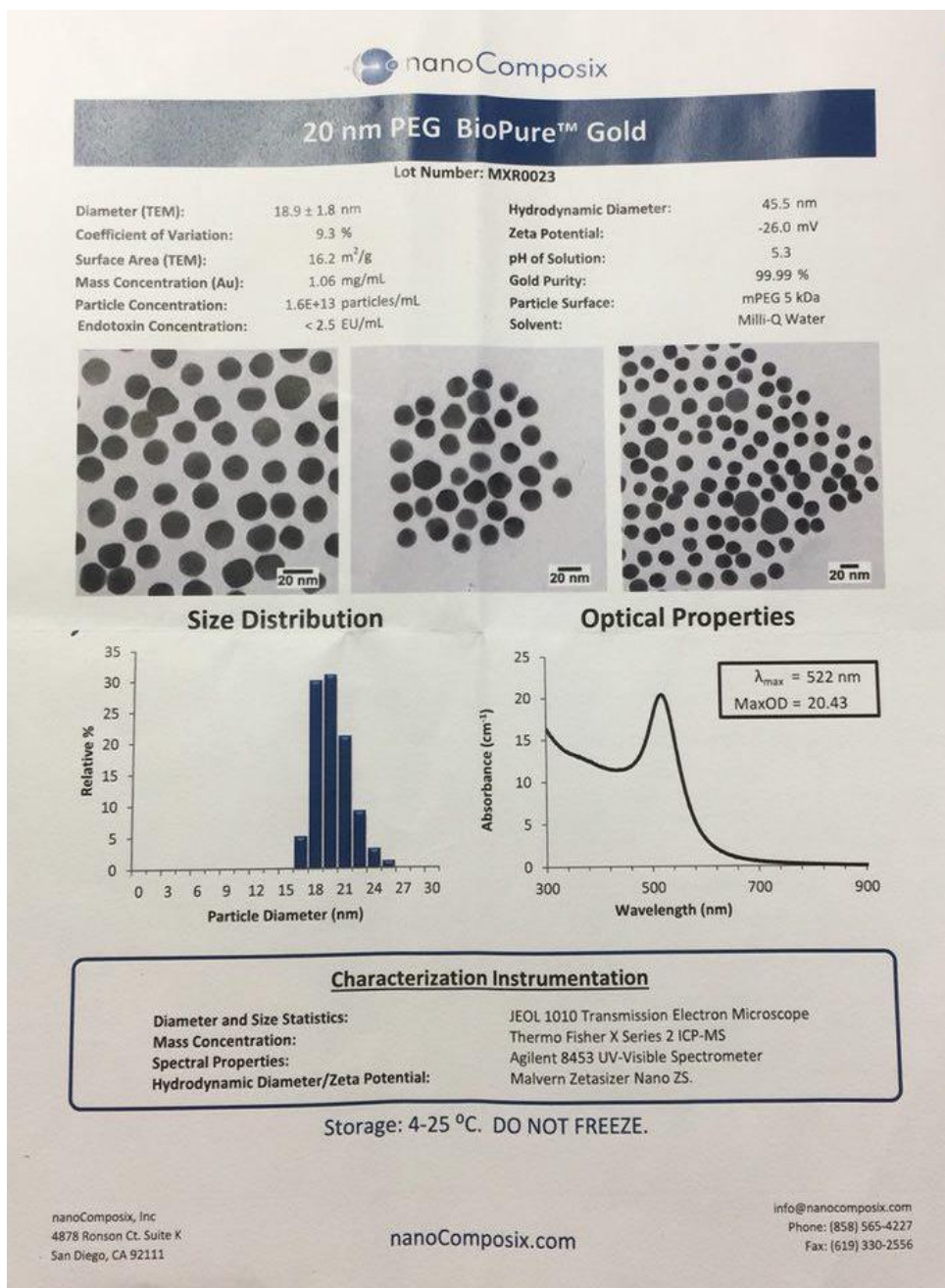
Supplementary Figure S2.4. Histological analysis of human liver sections. H&E stained human liver sections illustrating the uniform, compact internal structure of the liver with direct cell-cell contacts, defined outer perimeters, and zonation. Images reproduced from <https://www.lab.anhb.uwa.edu.au/mb140/CorePages/Liver/liver.htm> and <https://www.kenhub.com/en/library/anatomy/liver-histology>.



Supplementary Figure S2.5. Bile canaliculi observed in human liver tissue. Electron microscopy image illustrating the ultrastructure of hepatocytes forming a bile canalicular structure (denoted by *), and H&E stained liver sections imaged using epifluorescent

microscopy, with canaliculi denoted by black arrows. Image reproduced from <http://www.cellimagelibrary.org/home>

Supplementary Figure S3.1.



Supplementary Figure S3.1. Characterization data for 20nm AuNP kindly provided by supplier nanoComposix (San Diego, California). Analysis undertaken: TEM for morphology and size assessment, endotoxin contamination assessment, ICP-MS for mass concentration analysis, optical properties using UV-Vis, size and zeta potential using dynamic light scattering (DLS).

Supplementary Figure S3.2.

Batch Li50-2020-001 (LipImage 50 nm)

Manufacturing date	16/06/2020	Specifications
Operator for batch production	CEA-LETI/ Emilie Rustique	
Composition	Particle concentration: 95 mg/mL (9.5 %) Dye concentration (HPLC): 239.5 μ M (252 μ M/100 mg particle) Dispersion media: 154 mM NaCl + ascorbic acid 1.75 g/L	Particle concentration: 80-120 mg/mL Dye concentration: 239-293 μ M/ 100 mg particle
Storage information	Store at 4°C (room temperature possible for few hours) NEVER FREEZE (destroys particles)	
Batch quality control	Particle size (DLS): 52.2 nm Nanoparticle polydispersity (DLS): 0.102 Endotoxins : < 1UI/mL	Particle size (DLS): 45-55 nm Nanoparticle polydispersity (DLS): < 0.15 Endotoxins : < 1UI/mL

Supplementary Figure S3.2. Characterization data for LipImage™815, kindly provided by supplier CEA-LETI (France). Analysis undertaken: DLS for size and polydispersity index (PDI), HPLC for dye loading analysis and endotoxin contamination assessment.

Supplementary Figure S3.3.

SINTEF

Third round of samples for REFINE

Sample details for PACA particles

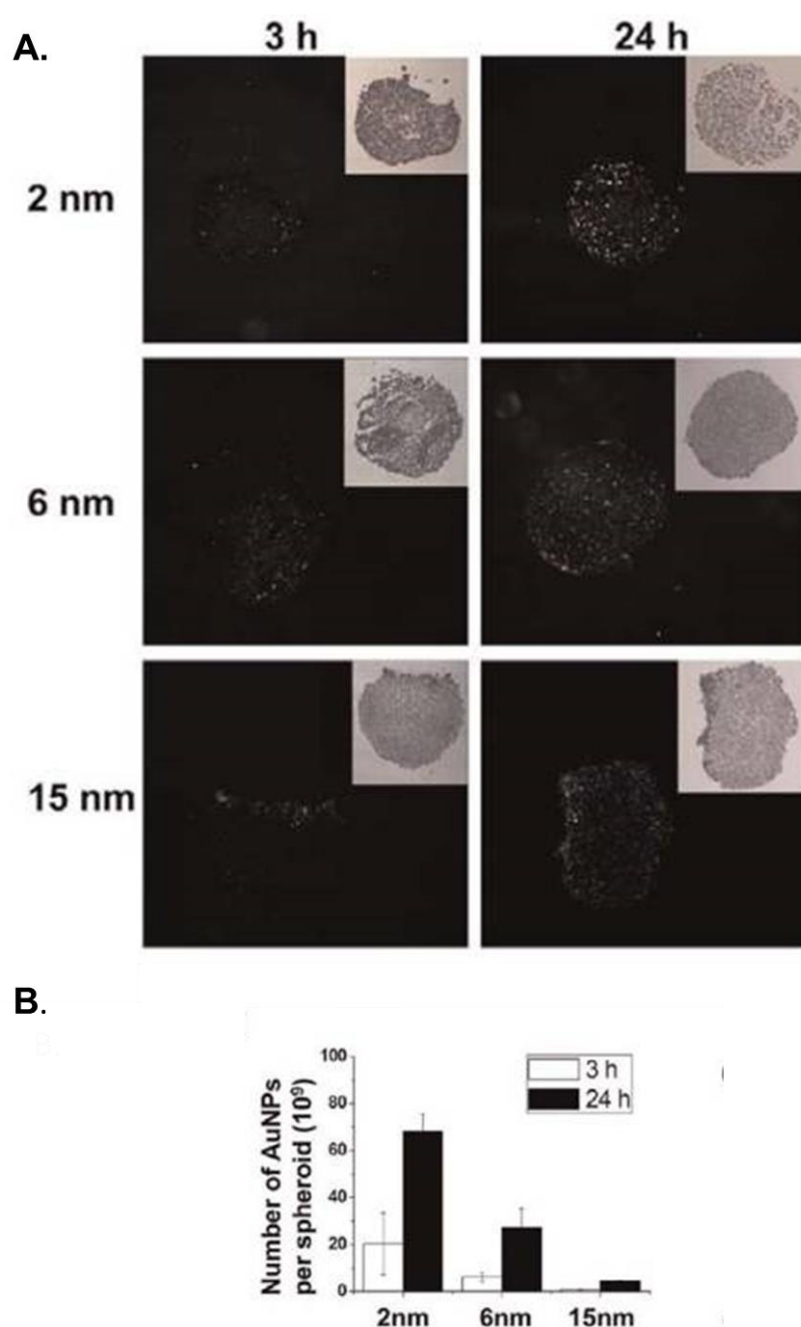
Batch name	Description	API	Dye	Size z-avg. (nm)**	PDI	Charge/Zetapotential (mV)**	Particle conc. in stock (mg NP/ml)	Drug loading (wt% of particle weight)*	Drug conc. in stock (mg/ml)*	Theoretical loading of dye (wt% of particle weight)	Suspension medium
REF-20-3 20-1	[REDACTED]										distilled water
REF-22-3 22-2	[REDACTED]										distilled water
REF-24-3 24-1	NR668-loaded PEBCA (PACA) nanoparticles	-	NR668	178	0.28	-3.6	1.05	-	-	0.43	1 mM HCl in sterile distilled water
REF-25-3	[REDACTED]										

* As measured by mass spectrometry at SINTEF.
 ** Measured by zetasizer in phosphate buffer pH 7

PEBCA = poly(ethylbutyl cyanoacrylate)
 CBZ = Cabazitaxel
 NP = Nanoparticle
 PDI = Polydispersity index
 NR668 = modified nile red (fluorescent dye)
 IR-780-oleyl = near-infrared dye

Supplementary Figure S3.3. Characterization data for NR668 PEBCA (PACA) kindly provided by supplier SINTEF (Trondheim, Norway). Analysis undertaken: DLS for size and polydispersity index (PDI), Zeta potential.

Supplementary Figure S3.4.



Supplementary Figure S3.4. Penetration of various sized AuNPs into tumour spheroids after treatment for 3 and 24 h.

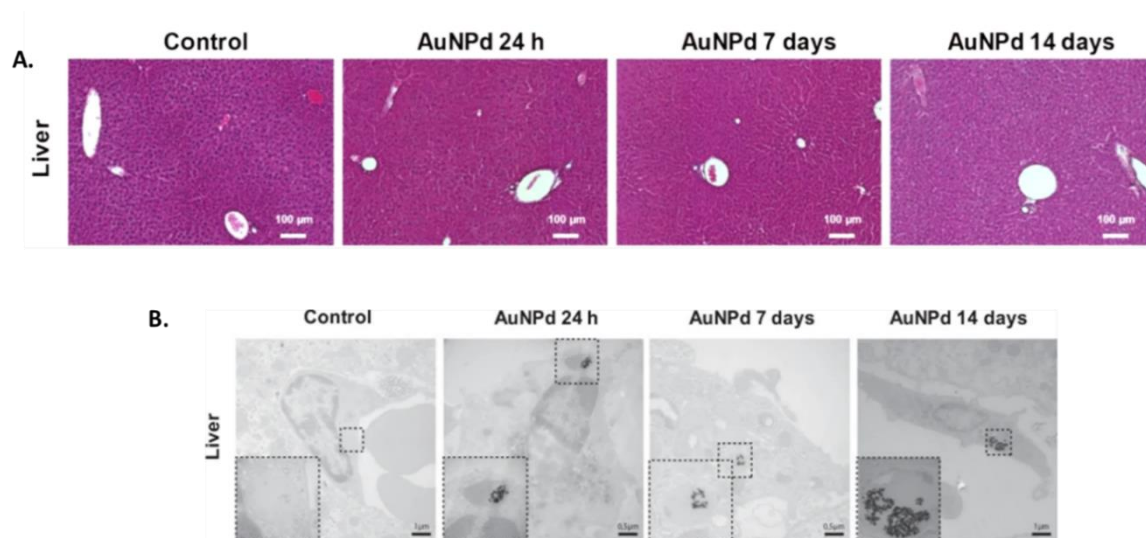
(A) Darkfield images of spheroids after culture with AuNPs of different sizes. (B) ICP-MS analysis of the number of Au nanoparticles in each treated spheroid. Figure reproduced from[377].

Supplementary Figure S3.5.

A study from Bailly *et al.* assessed the subcellular localization of AuNPs in the liver using TEM, 24 h, 7 d and 14d after injection, with AuNPs present in the liver of all animals to receive injections of AuNPs. Interestingly, AuNPs were present primarily in phagocytosing cells, i.e. Kupffer cells, with no AuNPs detected in other liver cells including hepatocytes or endothelial cells. Some AuNPs were found isolated in macrophages and Kupffer cells, however they preferentially agglomerated in clusters. Single AuNPs and AuNPs clusters were localised to the endo-lysosomal compartments.

Kupffer cells of control animals were found to have no AuNP internalised in them. No AuNPs were observed in the nucleus, cytosol or in other cytoplasmic organelles. No difference in subcellular location or accumulation was observed the different time points.

Kupffer cells of control animals were found to have no AuNP internalised in them, and no AuNPs were observed in the nucleus, cytosol or in other cytoplasmic organelles. No difference in subcellular location or accumulation was observed the different time points.



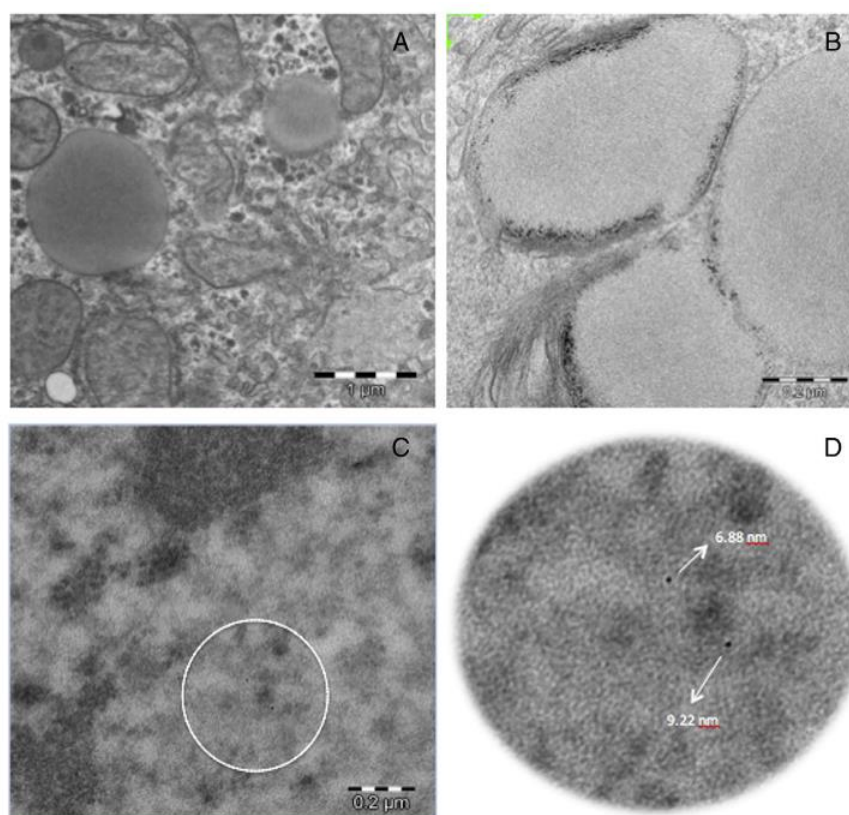
Supplementary Figure S3.5. Uptake of AuNP in the liver.

(A) Histological analysis of sections of mice liver 24 h, 7d, 14d after intravenous administration of a dextran coated AuNP (1 mg/kg) compared to the control group administered with vehicle alone. All sections were stained with hematoxylin-eosin. Scale bar = 100 µm. (B) Representative TEM micrographs of the subcellular location of AuNPd in hepatic tissues. TEM images of Kupffer cells in liver and macrophages in spleen of mice 24h, 7 and 14d after intravenous injection of AuNPd (1 mg/kg) compared to the control group administered with vehicle alone. Magnified views of the square area show endo-

lysosomal compartment containing clustered AuNPd in treated animals. Images reproduced from [368].

Supplementary Figure S3.6.

The distribution, bioaccumulation and toxicity of AuNPs *in vivo* is widely studied. One such a study from Lopez-Chavez *et al.* [383] has found that the liver has the highest accumulation of AuNPs, followed by the spleen. As illustrated below in Figure 3.14, TEM analysis suggests that AuNPs are taken up by hepatocytes, localized in the cytosolic lipid droplets (CLDs).



Supplementary Figure S3.6. TEM images from hepatocytes of rats treated with 10 nm AuNP.

(A) untreated control, (B, C and D) rats treated with 10 nm AuNP. (A) Subcellular structures with clean lipid droplets among them. (B) Lipid droplets with small AuNP inside them. (C) Nucleus with two AuNPs internalized. (D) Enlarged image from (C) illustrating the size of both internalized AuNPs. Scale bar (A) 1 μm and (B and C) 0.2 μm. Figure reproduced from [383].

Supplementary Figure S3.7.

AuNP uptake in Kupffer cells was observed in a study from Chen *et al.* Here, agglomerates of PEGylated AuNPs were primarily found enclosed in intracellular lysosomes of Kupffer cells (Supplementary Figure S3.7).

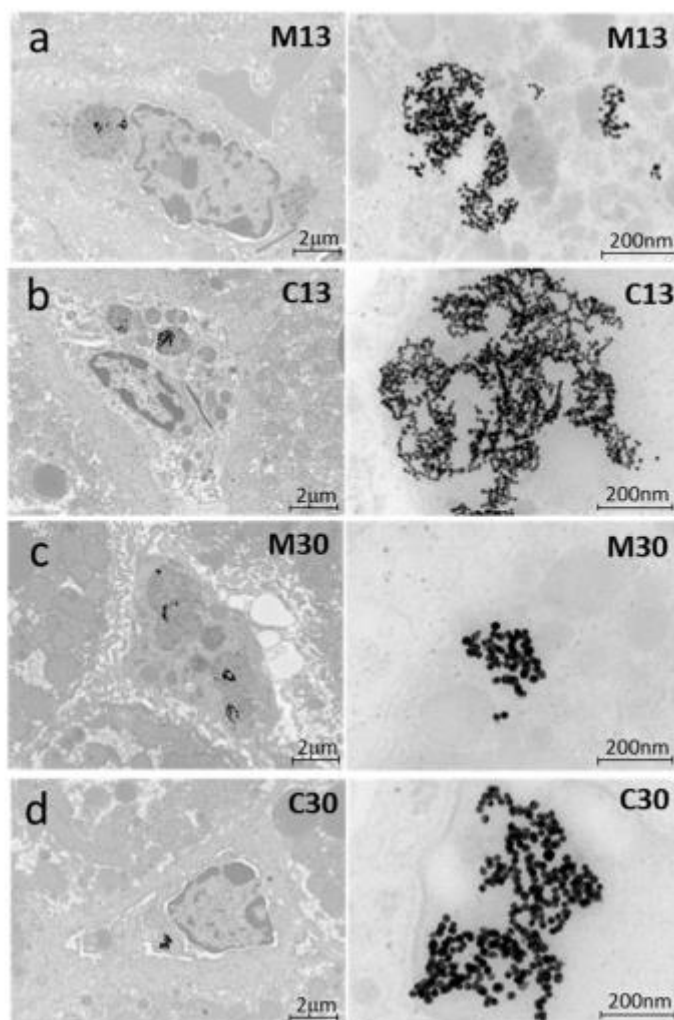
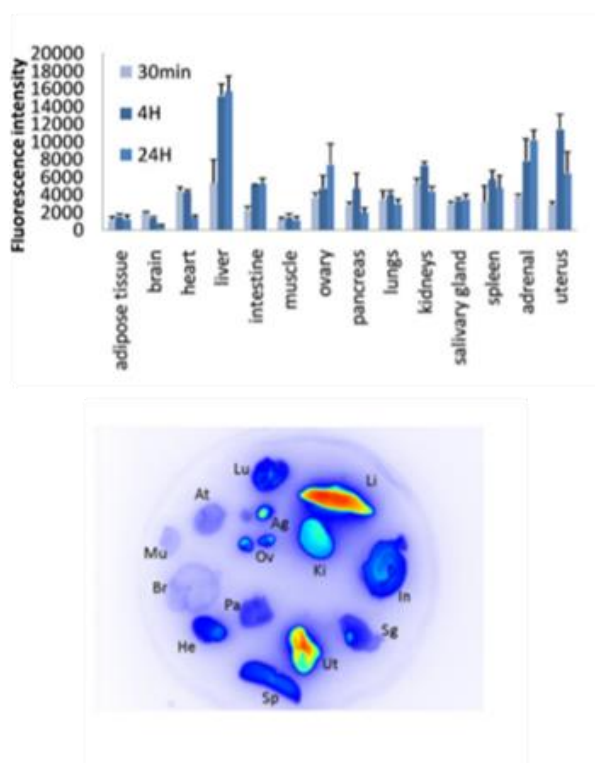


Figure 3.7. Agglomerates of PEG-AuNPs observed in liver Kupffer cells.

AuNPs observed in Kupffer cells at 3 months post 13 nm mPEG- or cPEG-AuNP injection (A and B), or by 30 nm mPEG- or cPEG-AuNP injection (C and D). Higher magnification images are shown at right. Figure reproduced from [384].

Supplementary Figure S3.8.

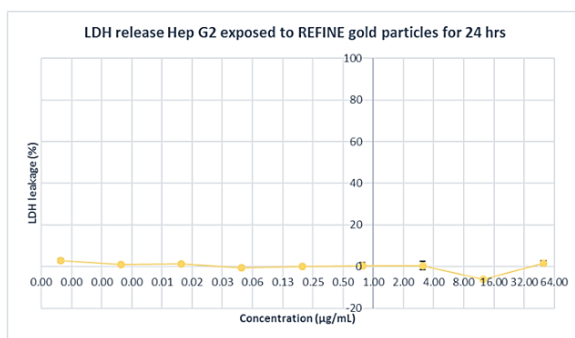


Supplementary Figure S3.8. Fluorescence biodistribution 24 h after injection of IR780-lipid dye.

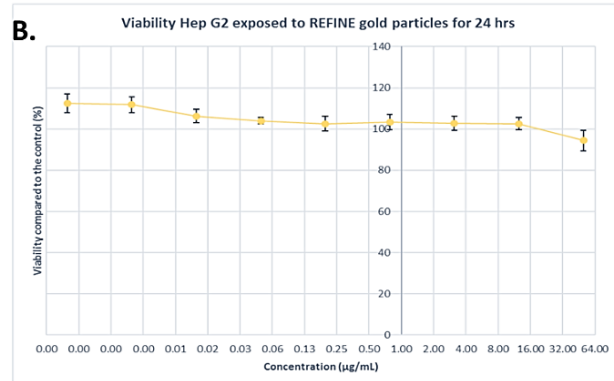
Fluorescence biodistribution of LipImage™ 815 in albino FVB mice. At, adipose tissue; Ag, adrenal glands; Br, brain; He, heart; In, intestines; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; Ov, ovaries; Pa, pancreas; Sg, salivary gland; Sp, spleen; Ut, uterus. Image reproduced from [34].

Supplementary Figure S4.1.

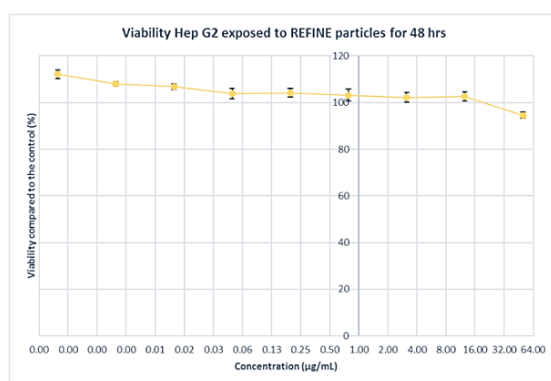
A.



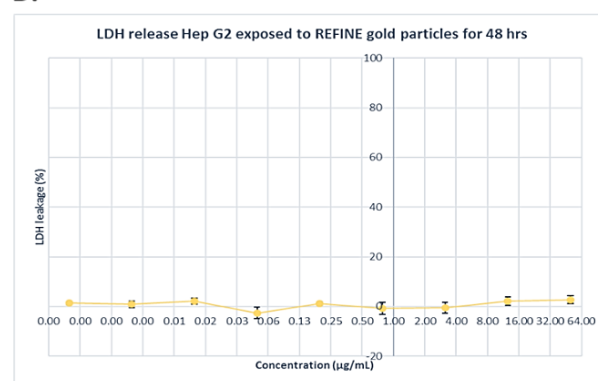
B.



C.



D.

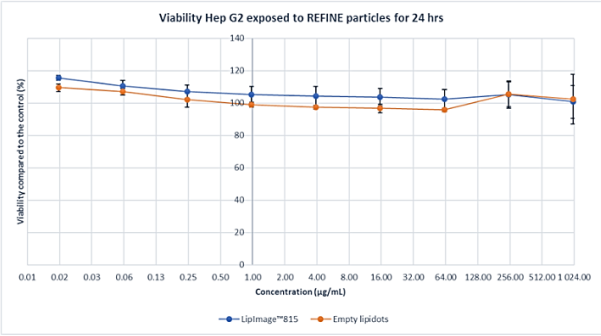


Supplementary Figure S4.1. Viability and cytotoxicity assessment of 20nm AuNP by Partner 1.

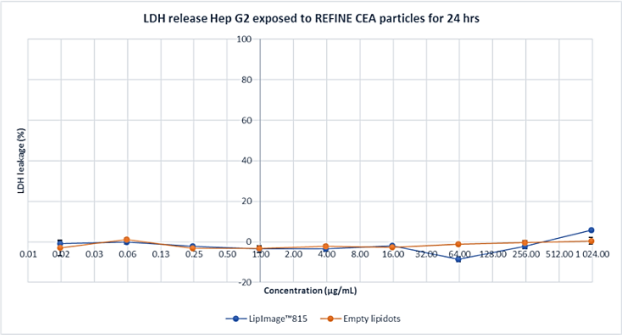
HepG2 cells were treated with increasing concentrations of AuNP for 24 and 48h before being assessed using the LDH and CTG assays.

Supplementary Figure S4.2.

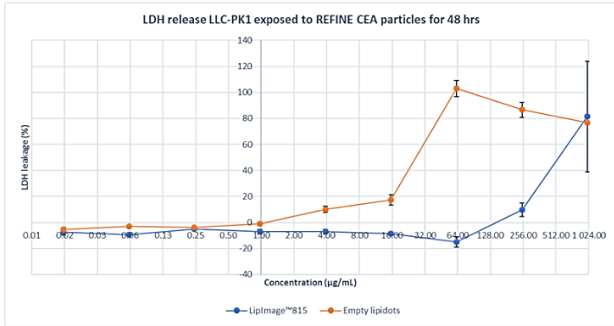
A.



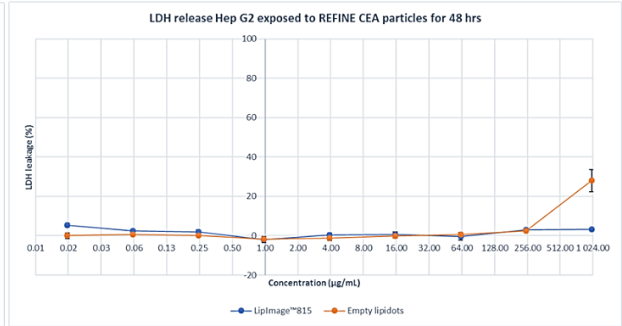
B.



C.



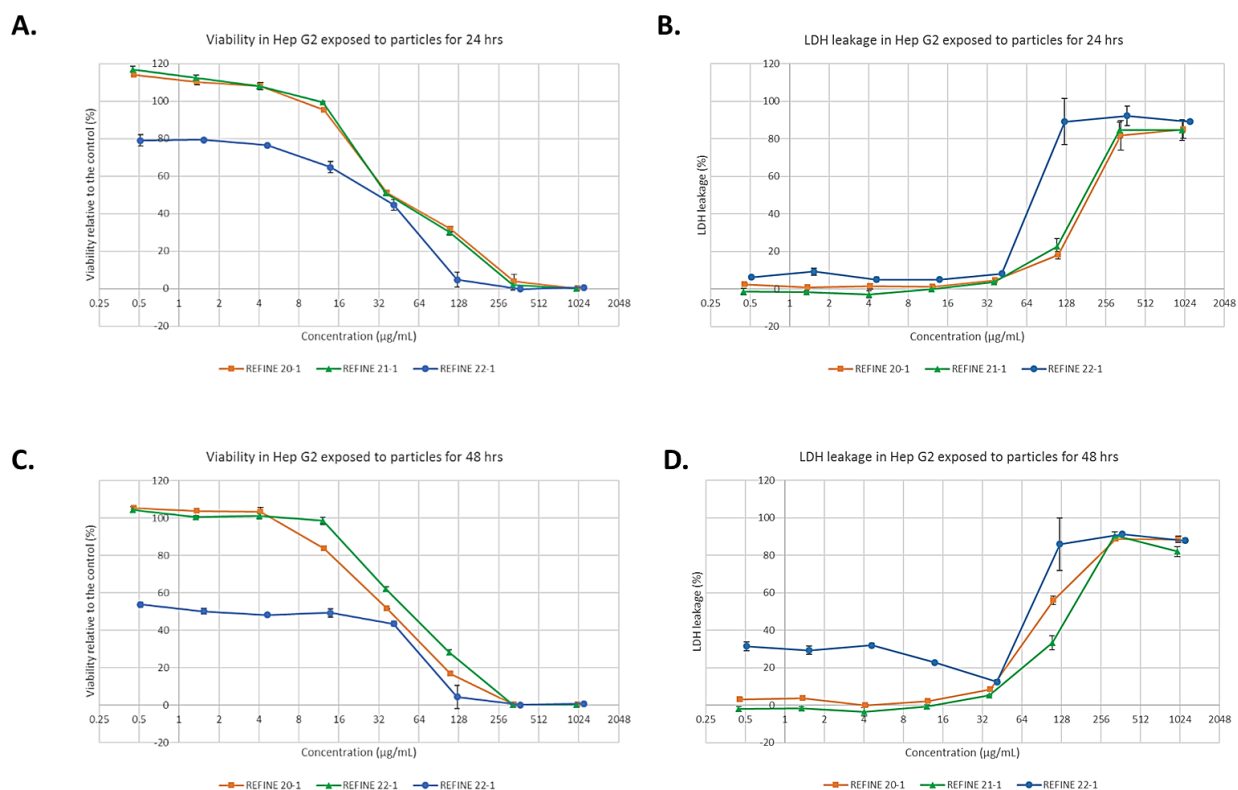
D.



Supplementary Figure S4.2. Viability and cytotoxicity assessment of LipImage™815 (blue line) by Partner 1.

HepG2 cells were treated with increasing concentrations of LipImage for 24 and 48h before being assessed using the LDH and CTG assay

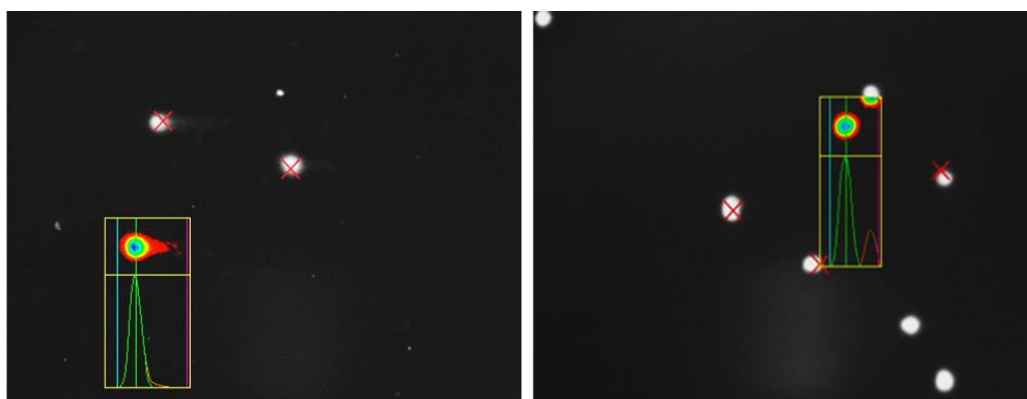
Supplementary Figure S4.3.



Supplementary Figure S4.3. Viability and cytotoxicity assessment of NR668 PEBCA (PACA) (green line) by Partner 1.

HepG2 cells were treated with increasing concentrations of PACA for 24 and 48h before being assessed using the LDH and CTG assays.

Supplementary Figure S4.4.



Supplementary Figure S4.4. Examples of negative scoring in the alkaline comet assay. Comets scoring using Instem CometIV software.



14Project: REFINE

Evaluating Nanomaterial induced DNA-strand break in HepG2 cells using the Alkaline Comet Assay

1. Introduction

The following protocol describes genotoxicity testing of human hepatocarcinoma (HepG2) cells following exposure to nanomaterials. Assessing DNA-strand break, i.e. the ability of a material to cause DNA damage, is undertaken using the Alkaline Comet Assay [496, 497].

1. Principle of the Method

This procedure details the steps for assessing the potential of nanomaterials to alter the genetic information of a cell and cause DNA damage. This is undertaken using the Alkaline Comet Assay, or single-cell electrophoresis, which measures DNA strand breaks at the single-cell level.

Briefly, HepG2 cells treated with varying concentrations of nanoparticles are lysed, combined with low melting point agarose and pipetted onto an agarose-covered pre-coated Comet slide. Once agarose has gelled, slides are submerged in ice cold lysis solution. Alkaline electrophoresis is then performed, with DNA unwinding taking place by placing slides in electrophoresis chamber and buffer for 20 min without current, followed by electrophoresis at a current of 24 V, 300 mA for a further 20 min. Slides are then removed from chamber, neutralized in TRIS buffer, rinsed with ddH₂O and dehydrated ethanol. Slides are then dried overnight, before staining with ethidium bromide solution and imaging using epi-fluorescent microscopy [498].

3. Applicability and Limitations (Scope)

The comet assay has achieved the status of a standard test, in the cascade of assays used to assess the safety of novel pharmaceuticals, nanomaterials and other chemicals [401]. Due to the adverse effects of DNA strand breaks to human health, assessing mutagenic potential of nanomaterials is a vital component of nano risk assessment.

The term genotoxicity refers to the ‘*processes which alter the structure, information content or segregation of DNA and are not necessarily associated with mutagenicity. Thus, tests for genotoxicity include tests which provide an indication of induced damage to DNA (but not direct evidence of mutation) via effects such as unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE), DNA strand breaks, DNA adduct formation or mitotic recombination, as well as tests for mutagenicity*’ [499].

This SOP details an *in vitro* method for evaluating genotoxicity in the HepG2 cell line following exposure to nanoparticles formulations. As sub-lethal nanoparticle concentrations are used, preliminary cytotoxicity experiments must be undertaken under identical conditions (i.e. cell line, culture medium etc.) to determine what a lethal dose is. Existing literature on the topic should also be reviewed. At present, all types of nanoparticle formulation are suitable for assessment using this protocol as long as there is no particle interference with nuclear DNA in its unwinding state, i.e., there should be no interfere with microscopic fluorescence readouts.”

Related Documents

Document ID	Document Title
OECD 489	<i>In Vivo</i> Mammalian Alkaline Comet Assay
	Handling of HepG2 cells

3.1 Equipment/Materials

1. Corning® Costar® 6 well flat bottom cell culture plates (Fisher Scientific, 10146810)
2. Superfrost Plus microscope slides (Fisher Scientific Ltd, J3800AMNT)
3. Incubator, 37°C with 5% CO² and 95% humidity
4. Hemocytometer or Automated Cell Counter
5. Large Vertical Electrophoresis Chamber (Thermo Fisher Classic Maxigel System CSSU2515)
6. Powerpack
7. Epi-fluorescent Microscope (e.g. Nikon Eclipse TE 300), 10X Objective
8. Appropriate imaging software, e.g. QCapturePro Program and QImaging Tool Version 7.0.0.8 (Exposure Acquisition Parameters: 000.154.603. Gain 3.870, Offset -1374)
9. Comet Assay IV software (Instem, United Kingdom)
10. GraphPad Prism software (GraphPad software Version 5)

3.2 Reagents

1. HepG2 (Human hepatocarcinoma cells) (HepG2 supplied by ATCC #HB-8065, Trinity Translational Medicine Institute, TCD)
2. TrypLE™ (Gibco, 12605-010)
3. Fetal Bovine Serum (FBS) (Sigma, F7524)
4. Penicillin-Streptomycin (Sigma, P0781)
5. PBS tablets (Fisher Scientific Ltd, 11435015)
6. DMEM, low glucose (Invitrogen™, 31885049)
7. Low Melting Point Agarose (Invitrogen, 16500100) CAS: 39346-81-1
8. Sodium Hydroxide (NaOH) (Sigma, S5881) CAS: 1310-73-2
9. Hydrochloric Acid (HCl) (Sigma, 30721) CAS: 7647-01-0
10. Na₂-EDTA (Ethylenediaminetetraacetic acid) (Fisher Scientific, 17892) CAS: 6381-92-6
11. TRIS (Sigma, 10708976001) CAS: 77-86-1
12. N-Lauroylsarcosine sodium salt (Sigma, L7414) CAS: 137-16-6
13. Ethyl methanesulfonate (EMS) (Sigma, M0880) CAS: 62-50-0
14. Ethidium Bromide (Sigma, E8751) CAS: 1239-45-8

15. Triton X-100 (Sigma, 93443) CAS: 9002-93-1
16. Dimethyl Sulfoxide (DMSO) (Sigma, D5879) CAS: 67-68-5
17. Sodium Chloride (NaCl) (Sigma, S7653) CAS: 7647-14-5
18. TiO₂ (NM101, JRC)
19. Virkon™ disinfectant (Fisher Scientific, 330013)

3.3 Reagent Preparation

3.3.1 Complete cell culture medium

Prepare complete low glucose DMEM cell culture medium by supplementing with FBS and penicillin streptomycin antibiotic. Remove 10% of total volume of medium from bottle, before adding 10% FBS and 1% penicillin streptomycin. Mix thoroughly.

3.3.2 Agarose.

Prepare 1.5% agarose in PBS. Dissolve 1.5 g agarose in 100 ml PBS. Boil in microwave until dissolved.

3.3.3 Low Melting Point (LMP) Agarose

Prepare 0.5% LMP agarose in PBS. Dissolve 0.5% LMP agarose in 100 ml PBS. Boil in microwave until dissolved.

3.3.4 NaOH

Prepare 5M NaOH solution by dissolving 200g NaOH pellets in 1L ddH₂O

3.3.5 EDTA

Prepare 0.2M Na₂-EDTA H₂O. Dissolve 18.612 g Na₂-EDTA H₂O in 250 ml ddH₂O

3.3.6 Lysis Buffer

2.5M NaCL, 100mM EDTA, 10mM TRIS, 1% N-Laurylsarcosine sodium salt

1. 146.1 g/L NaCL
2. 37.22 g/L Na₂-EDTA-2 H₂O
3. 1.21 g/L TRIS
4. 10g/L N-Lauroylsarcosine sodium salt

Dissolve 1-3 in 900 ml ddH₂O. Adjust pH to 10 with NaOH. Add 4, dissolve and add up to 1l with ddH₂O. Stir until clear.

3.3.7 TRIS neutralization buffer

Prepare 0.4 M TRIS solution in ddH₂O. Adjust to 7.5 pH with HCl. Dissolve 48.452 g TRIS in 1 l ddH₂O. Adjust to pH 7.5 with NaOH

3.3.8 Ethidium Bromide Staining Solution

Prepare 20 µg/ml ethidium bromide solution in ddH₂O. Dissolve 1 mg ethidium bromide in 50 ml ddH₂O.

3.3.9 Lysis Solution

(Prepared fresh on day of assay)

Prepare 75 ml lysis solution directly into glass cuvette. Wrap in foil and cool to 4°C.

66.75 ml lysis buffer (see 5.3.5), 7.5 ml DMSO and 0.75 Triton X-100.

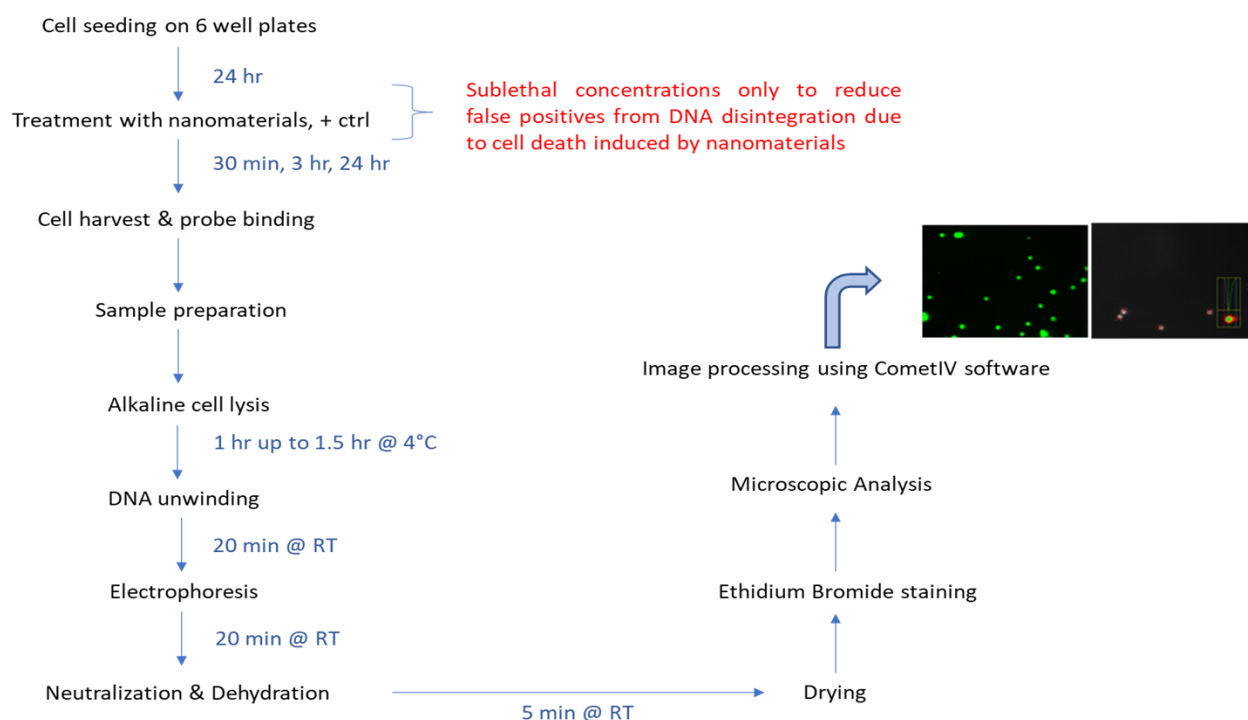
5.3.9 Electrophoresis Buffer

Prepare 1.25 l electrophoresis buffer. This is enough for 1 electrophoresis chamber and 20 slides. Cool to 4°C.

75 mL 5M NaOH and 6.25 mL 0.2 M EDTA. Add up to 1.25 l with ddH₂O.

Procedure

3.4 Flow Chart of the Measurement Procedure



3.5 Cell Handling

The HepG2 cell line was obtained from SINTEF (need batch number).

HepG2 cells are an adherent cell line maintained in low-glucose DMEM, supplemented with 10% FBS and 1% Penicillin Streptomycin. TrypLE is used for cell dissociation.

After thawing stock, cells are subculture up to 80% confluence (See Annex Fig. 1) is reached. HepG2 cells are routinely passaged 3 times a week and are maintained in T175 flasks due to the large cell volumes needed for the Comet Assay, at an approximate density of 15×10^6 in 100 ml at 80% confluence.

Subculturing

- Briefly, culture medium is removed, and cell layer washed twice with 10-15 ml pre-warmed (37°C) PBS.
- 5 ml of dissociation reagent (trypsin, TrypLE) is added and cells incubated for 5-10 min at (37°C), 5% CO₂.

- Flask is tapped gently to detach cell layer and cells resuspended in 10 ml culture medium to neutralize TrypLE.
- Cells are transferred to new culture flask containing fresh medium.
- Cells are incubated at 37°C and 5% CO₂.

6.3 Cell Seeding

1. HepG2 cells are harvested from flasks.
2. Cells are counted using hemocytometer and diluted to appropriate cell densities.
3. For the Comet Assay, 2.5×10^5 cells in 2.5 ml medium are seeded per well of each 6 well-plate. 6 wells are needed for nanomaterial treatments and 3 wells for controls (see Plate Design, Annex Fig. 2).
4. Plates are incubated for 24 hr at 5% CO₂, 37°C and 95% humidity, before treatment with nanomaterials.

Nanomaterial Treatment

Sublethal nanoparticle concentrations of test nanomaterials (prepared as detailed in 6.5) must be used for comet assay to reduce false positives from DNA disintegration caused by nanoparticle induced cell death. Prior to dilution, nanoparticles are characterized fully using appropriate characterization techniques. A positive nanoparticle control for DNA strand break is included, TiO₂ [398, 500-502].

3.5 Assay procedure

Cells are prepared in 6-well culture plates as described in section 6.3 and Fig. 1 in the Annex. Cells are treated with 6 sublethal nanoparticle concentrations, and controls; one genotoxic positive control (1M EMS), one negative control (media) and one nanoparticle genotoxic positive (10 µl TiO₂) control. Each test nanoparticle is diluted directly into cell culture medium. Each timepoint, i.e. 30 min, 3 h and 24 h are all treated at simultaneously (T0). All plates are dosed in the dark in flow hood and incubated in dark at 37°C, 5% CO₂ and 95% humidity.

Day 1 – Preparation and Cell Seeding

Dip-coat superfrost slides

1. Submerge superfrost slides in dd-H₂O and autoclave.
2. Use while warm but dry before dipping in agarose.
3. Boil 1.5% agarose solution in PBS.
4. Pour agarose (see 5.3.1) into 50 ml conical tube.
5. Dip slides in agarose three times (3 seconds each time, wiping excess agarose from back of slides each time) ensuring agarose layer is even on each slide.
6. Clean rear of slides with tissue and place horizontally on bench.
7. Let air dry overnight.

Seed cells on 6-well plates

1. Harvest cells and count as per section 6.2 and 6.3.
2. Add 2.5 ml of your cell suspension to each well.
3. Incubate plates overnight 5% CO₂, 37°C and 95% humidity.

Day 2/3 – Treatment with nanoparticles

1. Treat with 6 sublethal concentrations of chosen nanoparticles. For nanoparticle dilutions, nanoparticles are taken directly from vial and diluted into cell culture medium at appropriate concentrations in a sterile environment.
2. Three time points are analysed – 30 min, 3 h and 24 h. Each timepoint treated with NP suspensions at the same time.
3. Before dosing with particles, vortex all dilutions.
4. Remove cell culture medium and add 2.5 ml of particle in culture medium.
5. To negative control well, replace medium with fresh medium. To nanoparticle positive control (TiO₂), add particle directly in culture medium at a concentration of 10 µg/ml (See section 6.5).

Day 3 – Day of cell harvesting and comet assay

1. To positive control well, add 1M EMS (Ethyl methanesulfonate – CAS 62-50-0) control 30 min before end of culture period, for each of the three timepoints

EMS Control

Mix 44.4 µl ddH₂O with 5.1 µl EMS stock. Add 37.5 µl of this 1M EMS dilution to directly to EMS positive control well. Mix by swirling.

6. Incubate plates for a further 30 mins.

Prepare materials needed

1. Ice box to cool samples and lysis buffer (stored in fridge until use).
2. Eppendorf's for detached cells; labelled for sample dilutions/names, untreated control, positive EMS control, nanoparticle control.
3. LMP agarose prepared and stored at 37°C to prevent agarose jellifying too quickly.
4. Labelled cell culture tubes
5. Sticky labels labelled with nanoparticle and timepoint.
6. Labelled agarose coated slides, 1 per sample.

Cell harvest

1. After desired time points, remove cell culture medium and wash cells with pre-warmed PBS.
2. To detach cells, add 0.2 ml TrypLE per well and incubate for 5 min, followed by adding 0.3 ml culture medium to neutralize trypsin.
3. Transfer cell suspensions to labelled cell culture tube and place on ice.

Sample preparation and lysis

1. 160 µl LMP agarose is added to labelled Eppendorfs, followed by adding 40 µl of cell suspension.
2. Mix carefully by pipetting up and down, avoiding bubble formation.
3. 180 µl LMP agarose/cell mixture pipetted using yellow tips onto the pre-coated superfrost slides.
4. Cell suspension covered with cover slip and slides left to cure in fridge for 10 min (or up to 2 hr).
5. Repeat steps 1 – 4 for all other nanoparticle treatments.
6. Remove cover slip by gently turning it off slide and place slides back to back into pre-cooled lysis solution in glass staining jar.
7. Lyse for 1h (or up to 1.5 h) in fridge.

DNA unwinding and electrophoresis

1. Ensure electrophoresis chamber is level and fill with pre-chilled (4°C) electrophoresis buffer.
2. Fix voltage at power source to 24 V. Check current and adjust to 300 mA by changing buffer volume, i.e. if current below 300 mA add buffer, if current above 300 mA, remove buffer.
3. Remove slides from lysis solution and drain slides on tissue, being careful not to remove agarose.
4. Place slides in electrophoresis chamber, making sure labelled end of slide faces cathode.
5. Cover slides completely in electrophoresis buffer.
6. Allow DNA unwinding for 20 mins **without** current, then perform electrophoresis for additional 20 mins.

Neutralization, dehydration and ethidium bromide staining

1. After electrophoresis, take slides out of chamber, drain on tissue and put them back to back into glass chamber filled with TRIS neutralization buffer. Incubate for 5 min at RT.
2. Rinse slides with dd-H₂O by gentle shaking slides in beaker of water.
3. Dehydrate slides by putting them back to back in glass chamber filled with absolute ethanol. Incubate for 5 min at RT.
4. Take slides out, dry O/N leaning upright at RT.

Staining, slide analysis and image processing

1. After O/N drying, add 60 µl of 20 µg/ml ethidium bromide solution per slide.
2. Cover with cover slip.
3. Image slides under epifluorescence microscope, 10 X objective.
4. Appropriate imaging software depending on microscope used, e.g. Nikon TE300 and QCapturePro program & QImaging tool version 7.0.0.8 used for image acquisition.
5. Analysis of fluorescent images (ethidium bromide staining, Fig. 3 in Annex) and data mining carry out with Comet Assay IV software, supplied by INSTEM. 100 comets scored per slide, per time point, per nanoparticle treatment or control. Only comets that meet quality criteria scored, i.e. unsuitable comets not scored (See Q.C Section 7).
6. Data plotting carry out using GraphPad Prism software, Version 7.0.

3.6 Statistical Data Evaluation

- All experiments are conducted in triplicate.
- 100 comets are scored per slide with care taken to not score same comet twice (See Annex Fig. 3).

- Mean with standard deviation values calculated using GraphPad Prism Version 7.0. Analysis of variance (ANOVA) used for statistical analysis with significance level of at least or smaller 0.05. Underlining assumptions of test also analyzed.

3.7 Reporting of the Results

The collected data is compiled in a report, to be submitted in according to the timetable specified in the Deliverable list, EC-REFINE grant agreement, using appropriate reporting templates.

Critical information which should be reported includes the following: particle composition, particle concentration sample preparation method (i.e. dilutions), and cell seeding and nanoparticle dosing volumes. Assay conditions (i.e. for lysis, unwinding/electrophoresis and neutralization/dehydration) must be clearly detailed. Staining procedure, image acquisition parameters and method of processing data must be reported. All SOPs or related regulatory documents used should be detailed.

Quality Control and Acceptance Criteria

- 100 comets must be scored per comet slide, using a consistent pattern (shown in Annex Fig. 4).
- Mean tail intensity in untreated control must not exceed 8%.
- Care must be taken to ensure comets are not scored in overconfluent areas of comet slides as this leads to software falsely identifying comets (See Annex Fig. 5).
- Comets on the outer edges of slides must not be scored.
- ‘Ghost’ or ‘hedgehog’ comets, i.e. comets that only consist of a bright, distinct tail and no head, must be observed and reported as these are thought to indicate apoptotic cells, which may be indicative of non-sub lethality in nanoparticle dilutions (See Annex Fig. 5).
- Care must be taken to avoid scoring dust and debris. The comet assay is a multi-step protocol and there is a strong likelihood dust/debris will build up on slides.

Health and Safety Warnings, Cautions and Waste Treatment

- All cells and nanomaterial waste must be decanted into 1% Virkon and discarded into the appropriate waste container, according to local guidelines.
- Please refer to data sheets/MSDS for each reagent. Some reagents, e.g. ethidium bromide, pose potential health hazards and care must be taken with them.

- Protective equipment/clothing must be used/worn.
- Waste must be disposed of according to local regulations.

Abbreviations

DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
FBS	fetal bovine serum
HepG2	Human hepatocarcinoma cell line
LMP	low melting point
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
PBS	phosphate buffered saline
TRIS	trisaminomethane

10 Annex

10.1 Representative phase contrast image of confluent HepG2

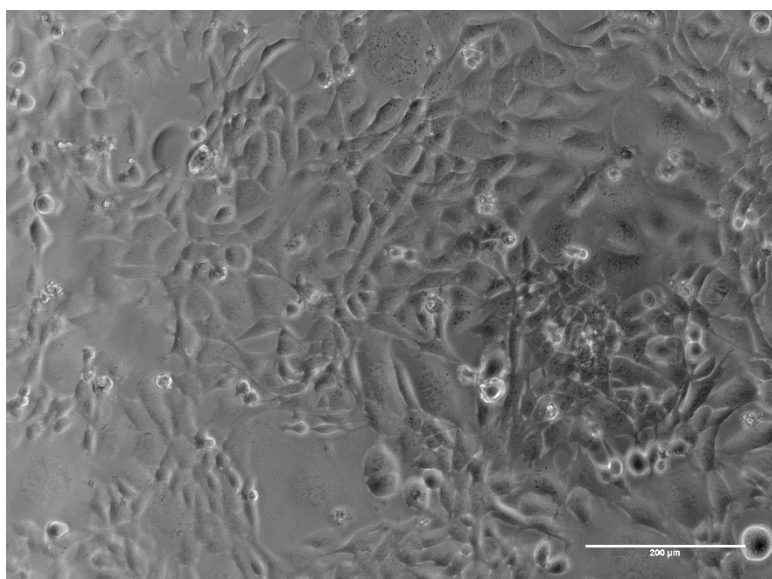


Figure 1. Confluent HepG2 cells imaged using Brightfield microscopy, as used to administer NP dispersions. Scale bar: 200 μm .

10.2 Comet Assay Plate Design

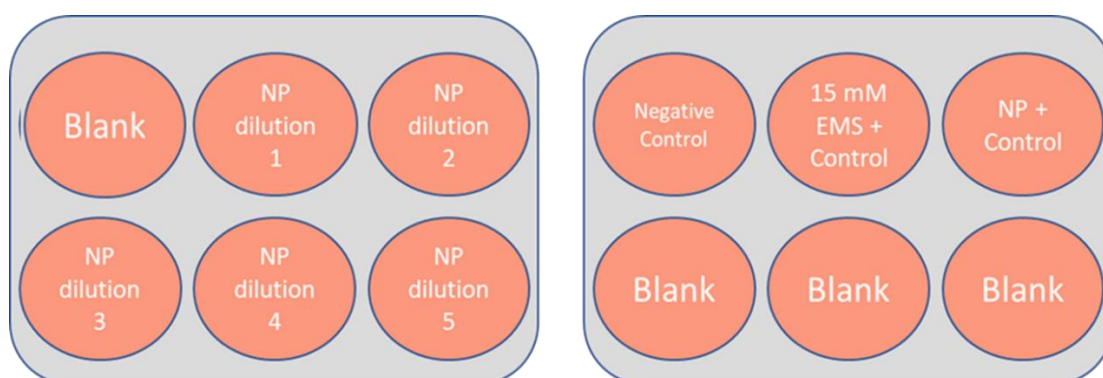


Figure 2. Plate design for alkaline comet assay.

5 nanoparticle dilutions, and 3 control groups – 15 mM EMS positive control, negative control (media), and suitable nanoparticle genotoxic positive control (e.g. 10 $\mu\text{g/mL}$ TiO_2).

10.3 Representative image of ethidium bromide staining and scoring

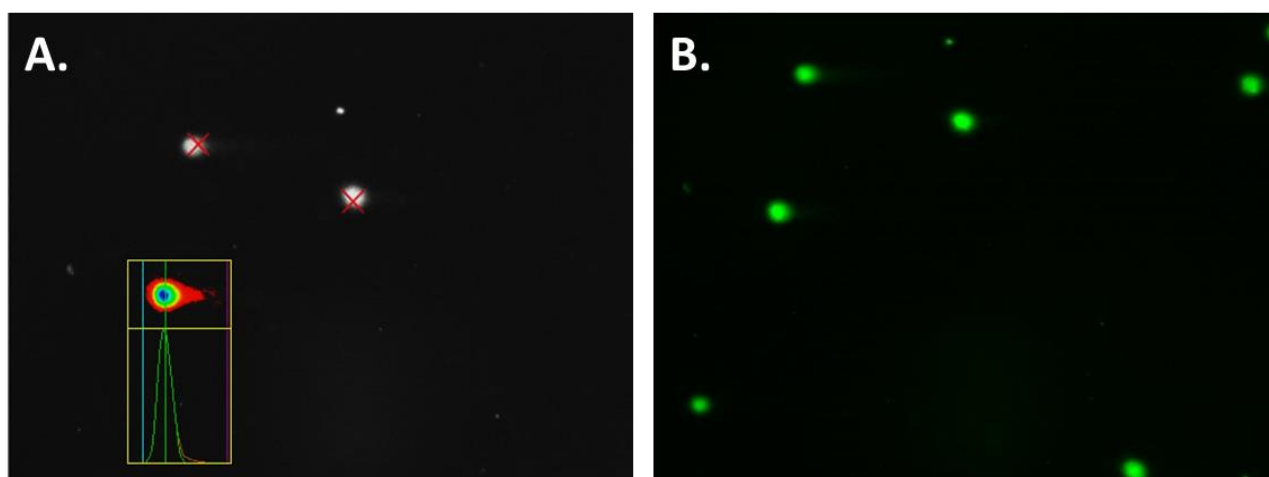


Figure 3. Representative images for (A) scoring of ethidium bromide stained negative control images with Comet Assay IV software, and (B) ethidium bromide cells.

Processed in green using ImageJ.

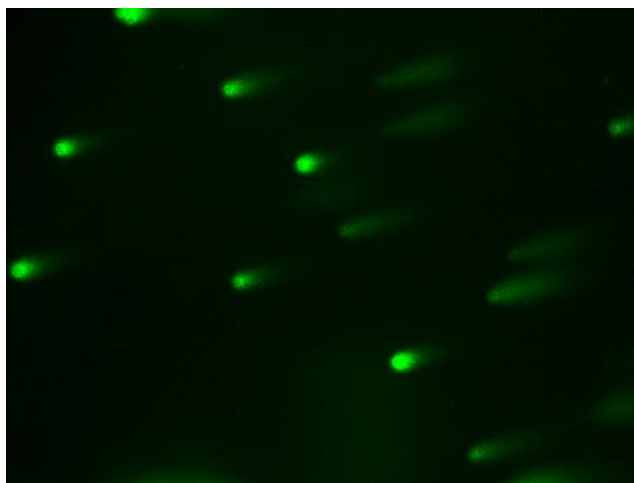


Figure 4. *Representative image of positive control images with ‘comets’ clearly visible.*

10.4 Comet Scoring Pattern

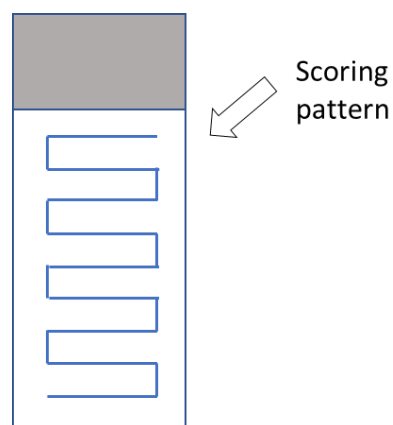


Figure 5. *Scoring pattern is implemented to avoid re-scoring the same cells/comets.*

10.5 Examples of comets which should not be score, i.e. outside acceptance criteria

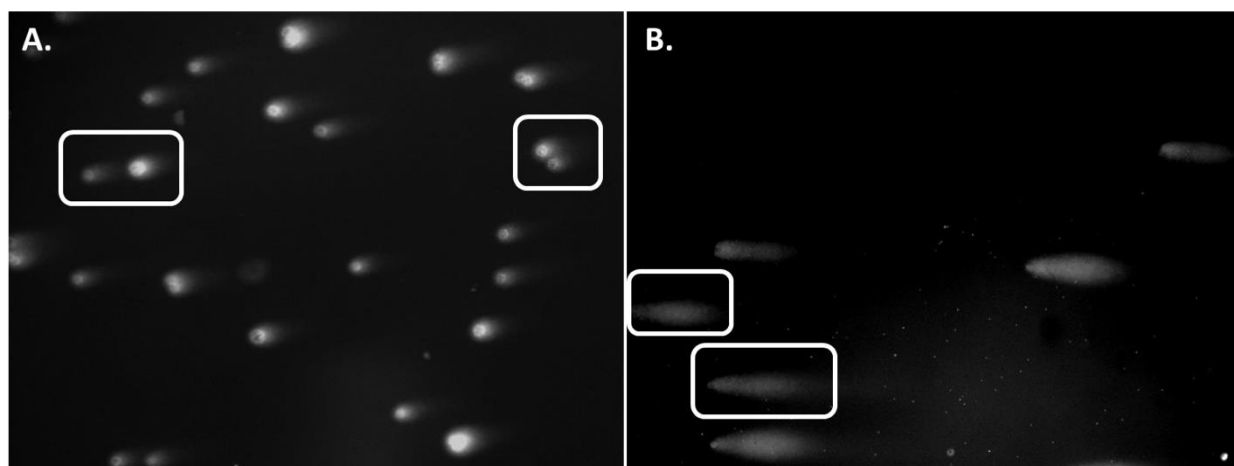
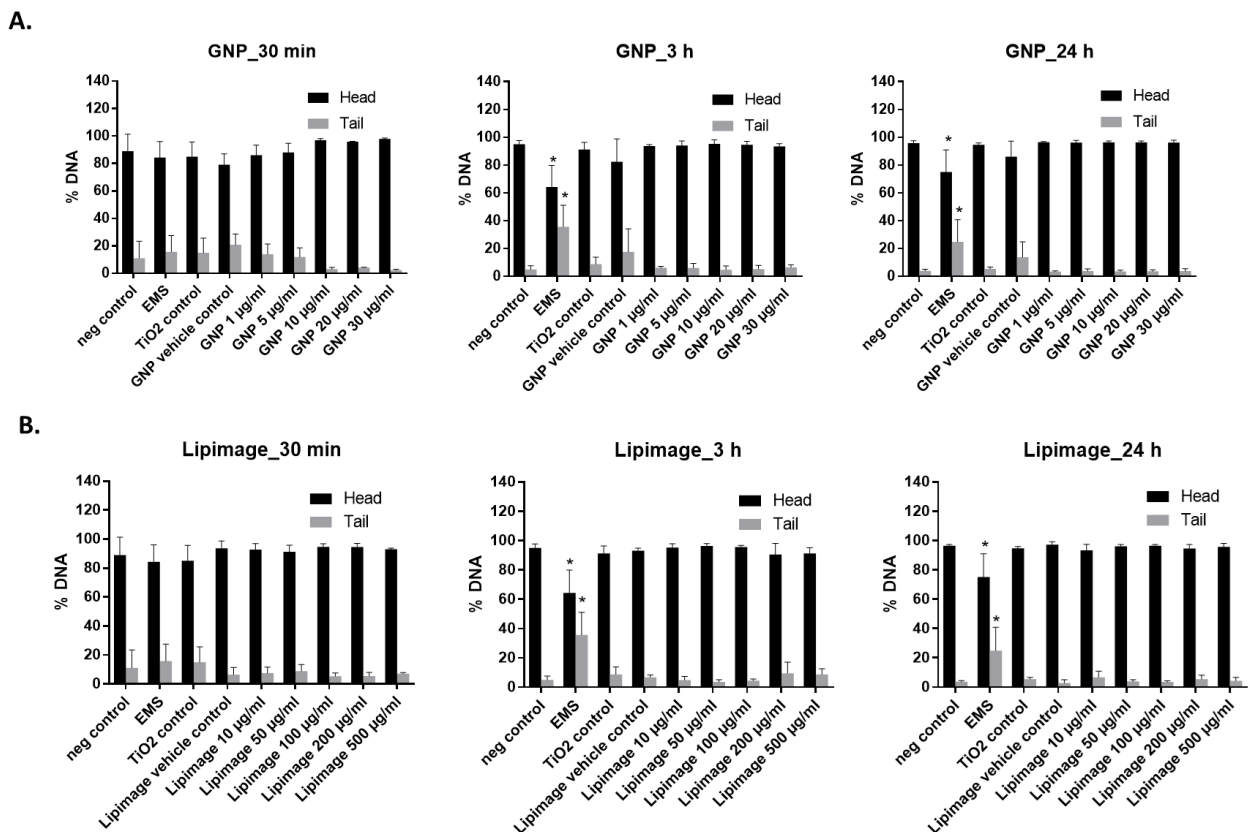


Figure 6. Comets which should not be scored.

- A. Cells in overconfluent areas of slide and B. 'Ghost' or 'Hedgehog' comets with no definite head and a long tail. These should not be scored as not representative of DNA damage and quantification not possible.

Supplementary Figure S4.6.



Supplementary Figure S4.6. Round robin alkaline comet assay data using AuNP and LipImage™815 from Partner 2.

HepG2 monolayers were treated with increasing concentrations of (A) AuNP and (B) LipImage™815 for 30min, 3 hr and 24 h before being assessed using the alkaline comet assay.

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