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In Vitro Three-Dimensional Liver Models for Nanomaterial DNA Damage Assessment

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Whilst the liver possesses the ability to repair and restore sections of damaged tissue following acute injury, prolonged exposure to engineered nanomaterials (ENM) may induce repetitive injury leading to chronic liver disease. Screening ENM cytotoxicity using 3D liver models has recently been performed, but a significant challenge has been the application of such in vitro models for evaluating ENM associated genotoxicity; a vital component of regulatory human health risk assessment. This review considers the benefits, limitations, and adaptations of specific in vitro approaches to assess DNA damage in the liver, whilst identifying critical advancements required to support a multitude of biochemical endpoints, focusing on nano(geno)toxicology (e.g., secondary genotoxicity, DNA damage, and repair following prolonged or repeated exposures).

1. Introduction

Throughout the years an increasing demand for smaller, lighter, faster, more adaptable, and durable technologies has led to the widespread adoption of engineered nanomaterials (ENMs) in a variety of industries such as food, cosmetics, textiles, manufacturing, electronics, energy, and the biomedical field. Nanotechnology had an estimated global market worth $48.9 billion in 2017 and is projected to reach $75.8 billion by the end of 2020; with the latter three industries (electronics, energy, and biomedicine) sharing over 70% of the global nanotechnology market.[1] Nanomaterials are defined as a natural, incidental, or manufactured materials containing particles in an unbound, monodispersed state, or as an aggregate/agglomerate where 50% or more of the particles possess one or more external dimensions in the size range 1–100 nm.[2] Nanotechnology is based on utilizing materials with pre-existing beneficial properties in bulk (>500 nm) and enhancing their physico–chemical properties via an increase in surface area to volume ratio by manufacturing these materials into particles of 1–100 nm in size.[3–5] However, the novel size specific physico–chemical properties (e.g., shape, size, crystal structure, composition, and surface charge) that enable ENMs to be associated with their advantageous applications are concomitantly causing heightened concerns regarding their potential adverse and unpredictable effects upon the environment and human health.[3,6] With the continued manufacturing, integration, and extensive application of ENMs, the risk of release into the environment and human exposure increases. Human ENM exposure occurs through four primary routes; dermal, inhalation, ingestion, or injection. Once ENMs have entered the body, they can undergo systemic translocation if they are able to traverse the biological barriers and enter circulation. This often results in multiple sites of deposition affecting various, secondary organ systems such as the liver, kidneys, and spleen.[7–12]

After inhalation, ingestion, and systemic administration through injection, it has been shown that ENMs deposit and can accumulate in the liver.[13–18] Hepatic toxicology is key when considering both chemical and ENM exposure, as the liver is vital for maintaining metabolic homeostasis and detoxification of both endogenous and exogenous substances.[19] For example, the liver possesses a higher mononucleated phagocytic system than that found in most other tissues or even the blood. This was illustrated when almost all of the administered dose of radiolabeled [48V] titanium dioxide (TiO_2) ENMs injected intravenously to healthy, female Wistar–Kyoto rats was directed straight to the liver for clearance.[20] However, the alveolar–blood-barrier and gut barrier act to reduce the rate of translocation into

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systemic circulation, with only 4.3% and 0.6% of the administered ENM dose entering the blood stream after 1 h. Although, the aim is to reduce the ENM load on surrounding tissues, this incidentally causes greater retention of the ENMs over extended periods of time.[20] It is becoming more apparent that translocation to secondary organs following inhalation or ingestion is low with <1% of the insoluble ENMs reaching secondary organs. The liver has been identified as a secondary organ that is highly susceptible to accumulation of ENMs and may result in liver damage and disease upon prolonged exposure.[20–22] Recently, Modrzynska et al. illustrated this by exposing 324 young adult female mice (C57BL/6) to 3.24 mg mL^{-1} of 10 nm TiO_2, 13 nm cerium dioxide (CeO_2), and 14 nm carbon black via three different exposure regimes; intratracheal instillation, intravenous injection, and oral gavage.[23] All three ENMs were found to translocate to the liver, primarily detected in the hepatic capillaries (e.g., sinusoids) and appeared to have been phagocytized by the liver resident macrophages, known as Kupffer cells. Even 180 days after exposure these ENMs remained within the liver tissue. The frequency and size of the ENM aggregates found in the liver tissue varied depending on the exposure method, suggesting not all the material is cleared easily from the liver.[23] Miller et al. also demonstrated this element of bio-persistence in 14 healthy, human males, whereby 3.8 nm gold (Au) ENMs inhaled over a 2 h period remained within the blood stream 3 months later, even after being detected in the blood of some volunteers as early as 15 min after exposure.[24] These findings suggested that ENM translocation into systemic circulation occurs rapidly and with no evidence of a time-dependent increase in Au ENMs present in the blood, indicating that the rate of translocation may be balanced by the rate of clearance.[24] However, slow incremental accumulation and bio-persistence in systemic tissues poses a potential threat to human health and the environment in the long-term. Evidence for this was demonstrated by the significant increase in DNA strand breaks and hepatic genotoxicity detected, only at later time points (28 and 180 days), following inhalation exposure to carbon black.[23]

Experimental toxicology has focused on supporting "The 3 Rs" directive to reduce, replace, and refine in vivo animal-based experiments with the aim to develop and utilize advanced in vitro-based systems as more ethical, cost effective, high-throughput alternatives for hazard characterization and risk assessment of chemicals and drugs. Regulations introduced by the EU Registration, Evaluation, Authorization and Restriction of Chemicals stress the need for standardized,[25] next generation in vitro tests systems that can be trusted to provide predictive and reliable results. Since multiple studies have shown that ENMs are able to accumulate in the liver and can lead to hepatotoxicity upon long-term exposure, there is a need to enhance the longevity and realism of current in vitro liver models to accurately assess the hepatotoxic potential of nanomaterials. Ideally, these in vitro models need to emulate the physiological environment of the human liver, as well as remain functionally stable over longer periods of time to be able to support more realistic exposure scenarios (e.g., long-term single or repeated, low-dose exposures, sequential incubations in physiologically relevant simulant fluids, and the addition of multiple cell types). In addition, to fully reflect the in vivo situation, advanced multicellular 3D in vitro models are needed to recapitulate the complex, intricate organ structure and active metabolic function. This ensures a better understanding of the underlying mechanisms leading to liver injury in a natural exposure scenario. ENM exposure in the liver has been linked to the induction of lysosomal disruption, as well as mitochondrial disruption. The latter can lead to an imbalance in oxidative homeostasis, an inflammatory response involving the release of cytokines, recruitment of immune cells, and subsequent oxidative stress. All of which can result in reduced liver functionality, DNA damage, and cell viability.[26]

There are a wide range of 3D in vitro liver models available on the market to support chemical and drug toxicity screening, all with benefits and limitations as extensively reviewed by Lauschke et al., 2019 and van Grunsven, 2017. Based on these reviews, a number of these model test systems are designed in a manner that could deem them unsuitable for ENM associated DNA damage assessment in vitro. For example, the addition of matrices or scaffolds creates a barrier that ENMs may not be able to traverse, thereby preventing appropriate exposure of the test material to the target cells. This has the potential to result in dosimetry inaccuracies and uncertainty concerning the actual ENM concentrations applied to the culture. Another limitation is that current in vitro 3D liver models are often formed from static, fully differentiated cells (i.e., non-dividing cell models) which limits genotoxicity assessment; for example, it deems them unsuitable for use with the gold-standard cytokinesis-block micronucleus (CBMN) assay (OECD TG487), where actively dividing cells are a necessity. Furthermore, there is a distinct lack of 3D in vitro liver models with the capability to evaluate secondary genotoxicity mechanisms induced by a chronic inflammatory response, which is recognized as a key mechanism underlying DNA damage induction associated with ENM exposure in vivo.[27–29] The standard in vitro 2D and 3D monoculture test systems for genotoxicity evaluation are only capable of detecting primary genotoxicity, thereby overlooking a key DNA damage mechanism associated with ENM exposure that occurs in vivo. Therefore, in this review we focus our discussion on the evaluation of currently available in vitro liver models and their suitability for ENM-induced DNA damage screening.

2. Liver Anatomy, Physiology, and Adverse Outcome Pathways Associated with Hepatic Engineered Nanomaterial Exposure

The liver consists mostly of hepatocytes (60%), and other non-parenchymal cell types, that influence the response toward ENM accumulation in the liver. Non-parenchymal cells include Kupffer cells (phagocytes), stellate cells (lipocytes), and liver sinusoidal endothelial cells (LSECs) which form the walls of the sinusoids (50–180 nm wide blood vessels) that carry blood throughout the liver.[30,31] These cells are organized into a hexagonal shaped liver lobule with the central vein in the middle, and the sinusoids radiating out to the vertices where the portal triad (including the portal vein, hepatic artery, and bile duct) is located, as indicated in **Figure 1**. Liver lobules are structured with a vast sinusoidal network to allow for the free transfer of oxygen, nutrients, and waste products between the hepatocytes

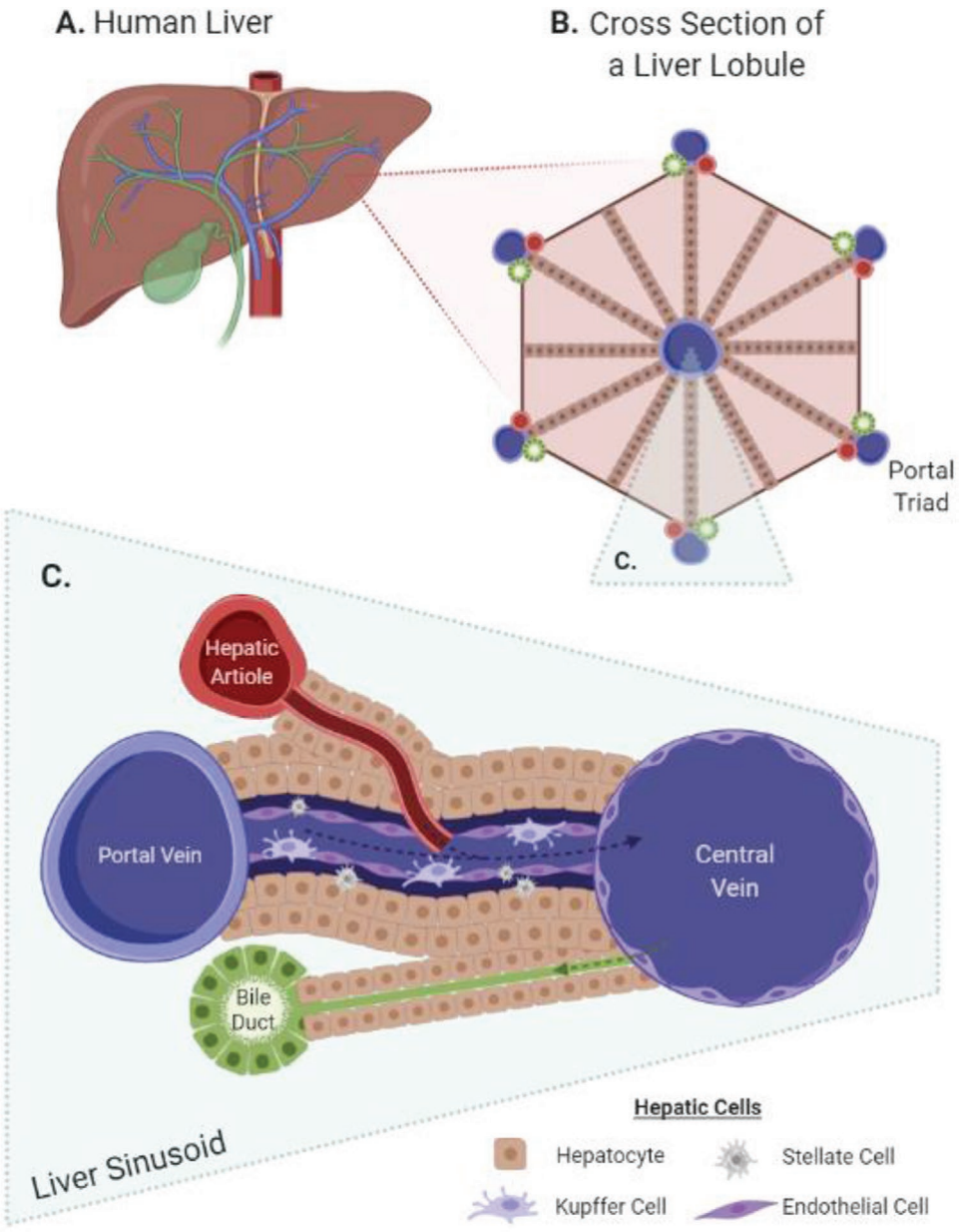

Figure 1. Diagrammatic representation of human liver physiology broken down from A) the liver itself to B) the hepatic lobules consisting of C) multiple liver sinusoids arranged in a hexagonal format. Created with BioRender.com.

and blood traversing along the sinusoid. This results in location dependent partitioning of cellular functionalization, known as "metabolic zonation".[31] As a result, the diverse cell populations and specific anatomical organization are crucial to maintaining a viable organ system and hepatic functionality.

The liver possesses the ability to repair and restore sections of damaged tissue following acute injury. However, prolonged exposure to ENMs may induce repetitive injury leading to chronic liver disease, whereby the regenerative capabilities are impaired, and the hepatocytes begin to undergo cell death as a result of inflammation.[19,32] There are four main adverse outcomes associated with hepatic injury (**Figure 2**), with liver inflammation, fibrosis, and cancer identified as adverse outcomes relevant to chemical and ENM toxicological risk assessment, and thus are key focal points when developing hepatic models in vitro. In 2012, the Organisation for Economic Co-operation and Development (OECD) launched a new initiative to develop a framework to assess these adverse outcomes and the key events leading up to them. Adverse outcome pathways (AOPs) are substance-agnostic and strictly describe the sequence of biological events connecting an exposure to an adverse outcome. In other words, AOPs do not describe the mode of action of a specific substance, even if a substance is linked to AOPs by their ability to provoke the molecular initiating events. A specific substance may also be used to provide empirical evidence for the existence of an AOP; for example, two AOPs describing liver pathologies have been linked to ENM as stressors.[33] AOPs have the potential to support systematic

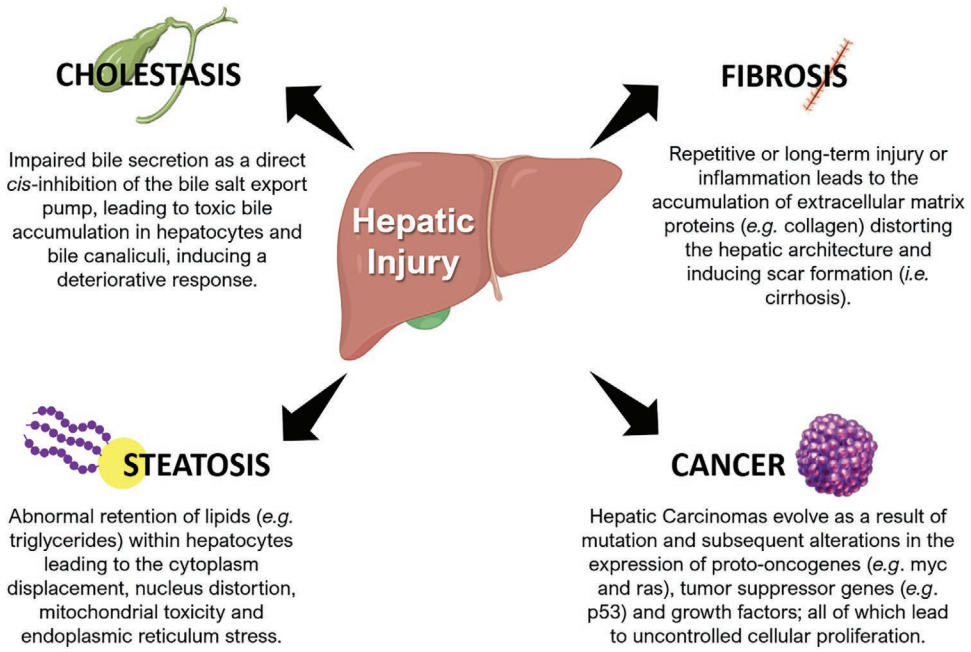

Figure 2. An overview of the four main AOPs associated with hepatic injury. Created with BioRender.com.

review and integration of highly diverse data types, including information from novel in silico and in vitro assays, which are generally not employed by risk assessors.[34] There are currently 20 AOPs describing diverse liver pathologies in the AOP-Wiki, 17 of which are focused on liver pathologies in humans, including hepatotoxicity (two AOPs), liver injury (two AOPs), non-alcoholic steatohepatitis (one AOP), steatosis (five AOPs), cholestasis (one AOP), fibrosis (two AOPs), and cancer (four AOPs). One of the AOPs (AOP: 38 for liver fibrosis) has been endorsed by the OECD (indicating high quality) and is one of the most well-developed AOPs to date.[33] In addition, three AOPs for liver cancer are under review by the OECD (AOP: 41, 46, and 220) and four are in development under the OECD work plan (AOP: 27 [cholestasis], 37 [cancer], 130 [hepatotoxicity], and 144 [fibrosis]). AOPs provide a useful tool for predicting specific mechanisms behind hepatotoxicity and should be considered as flexible constructs open to continual development and refinement as more relevant data is released.[35]

2.1. Liver Fibrosis

Prolonged incidence of hepatic apoptosis and/or necrosis often leads to the formation of hard scar tissue in place of healthy soft liver tissue; a process more commonly known as liver fibrosis. Liver fibrosis is mediated by a plethora of growth factors and cytokines released by damaged or dying hepatocytes. This leads to the activation of integrated signaling cascades, which are responsible for the phenotypic transformation of quiescent vitamin-A storing hepatic stellate cells into contractile, proliferative, and fibrogenic myofibroblast-like cells.[32,36] Thus, the majority of in vitro liver fibrosis models are found to comprise of hepatic epithelial cells cocultured independently with hepatic stellate cells or with the addition of other non-parenchymal cell types (e.g., human Kupffer cells and LSECs). Multiple models have been developed in an attempt to recreate a pro-fibrotic environment using the introduction of hepatic stellate cells, which have been acknowledged as the leading scar forming cell type in most liver injuries.[32,37] It has been frequently found that the greater presence of hepatic stellate cells is commonly linked to the proliferation of hepatoma cells and enhanced tumor metastasis.[38,39]

2.2. Liver Inflammation

In the liver, host defense and innate immune response is mediated by the resident macrophages, Kupffer cells.[40,41] Kupffer cells are localized within the hepatic sinusoid and account for 15% of the total hepatic cell population, resulting in a 1 to 4 ratio of Kupffer cells to hepatocytes. They are active members of the mononuclear phagocytic system, that serve a vital role in the mediation of inflammatory response, immune-mediated hepatotoxicity, liver injury, regeneration, and prevention of liver disease.[42] Kupffer cells function primarily in the ingestion and degradation of both endogenous and exogenous xenobiotics as well as senescent cells, cell debris, and other particulate matter present in the portal blood.[42] Furthermore, they are known to phagocytose pathogens, recruit neutrophils, and release both (pro-)inflammatory (e.g., IL-1β, IL-6, and TNF-α) cytokines and (pro-)fibrotic markers (e.g., TGF-β).[42,43] Kupffer cells are replete with toll-like receptors (i.e., TLR4, TLR2, TLR3, and TLR9) and have been shown to release inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12, IL-18, and IL-10 on activation with lipopolysaccharide.[41,44,45] The release of these factors influence and regulate the phenotypes of neighboring hepatocytes and other non-parenchymal cells (e.g., stellate cells and endothelial cells), by triggering signaling pathways that regulate

cell proliferation, viability, and cell death as well as functional cell changes (e.g., hepatocyte drug-metabolizing enzyme activities).[42,46,47] The presence of Kupffer cells is known to be a key determinant of liver fibrosis, as there is a finely balanced autoregulation between the release of (pro-)inflammatory and inflammatory mediators which in certain cases can exacerbate the initial damage.[45] Subsequently, Kupffer cells can facilitate chronic inflammation and liver fibrosis as a result of oxidative stress induced by cytokine release; a known adverse outcome of ENM exposure.

ENMs can act as exogenous reactive oxygen species (ROS)/reactive nitrogen species (RNS) inducers that can influence intracellular calcium concentrations, activate (pro-) inflammatory transcription factors (e.g., nuclear factor kappa β [NF-kβ]) and modulate cytokine production via the production of free radicals contributing to an imbalance in the redox homeostasis of the cell.[48,49] It has been hypothesized that the increased surface area and presence of pro-oxidant functional groups on the ENM surface are responsible for their enhanced ability to produce ROS.[49,50] ENM-related oxidative stress has been reported to incite a series of pathological events from inflammation and fibrosis to genotoxicity (i.e., chromosomal aberrations including single and double strand breakages and loss/gain of whole chromosomes, point mutations, and epigenetic changes) and carcinogenesis.[5,48,51] It is widely accepted that ENMs can both directly and indirectly affect intracellular ROS and/or antioxidant (e.g., glutathione and *N*-acetyl-cysteine) levels which are often linked with a (pro-)inflammatory response.[52–54]

Consequently, not only do these resident macrophages play an important anti-inflammatory role, but their presence within an advanced in vitro model allows for secondary genotoxicity to be assessed. Therefore, models which include multiple cell types, specifically Kupffer cells, in a 3D environment would be more beneficial to accurately assess the comprehensive effect of ENM exposure, accumulation within liver, and the progression toward hepatic adverse outcomes. In addition, it has been shown that hepatic metabolism, such as glutamine and albumin synthesis, cytochrome p450 enzyme activation, xenobiotic metabolism, and urea synthesis are often enhanced when cultured with macrophages.[55] Kostadinova et al. further illustrated this when culturing hepatocytes with non-parenchymal cells (i.e., Kupffer cells, endothelial cells, and stellates cells) which not only displayed increased albumin synthesis, but also increased transferrin, fibrinogen, and urea production. Furthermore, the macrophage coculture alone had increased CYP450 inducibility, and was more responsive to inflammatory stimuli and hepatotoxins when compared to the monoculture model.[56] This not only indicates that the inclusion of non-parenchymal cells may more realistically recapitulate liver structure, function, and response to toxins, but may also provide the necessary improvement in the predictive value of in vitro liver models.

2.3. Liver Cancer

Commonly, the etiology of multiple liver pathologies stems from liver fibrosis impeding liver functionality via the distortion of hepatic architecture and blood flow.[32] Later stages of liver fibrosis are often associated with the development of cirrhosis and hepatocellular carcinoma; the second leading cause of cancer-related deaths globally, with a 55% increase in liver cancer mortality rates in the UK alone over the last decade.[32,57–59] Liver cancers are categorized as a heterogeneous group of malignant tumors with different histological features. Tumor progression can be subdivided into different "modes of action" (MOA) with chemicals being defined as having a non-mutagenic or mutagenic MOA. Aflatoxin B1 for example, is a highly potent hepatic carcinogen, found as a contaminant of food, and is known to adopt a mutagenic MOA (AOP 46, AOP-Wiki). Metabolism of aflatoxin B1 results in the formation of the metabolite aflatoxin B1-8,9-epoxide, that can induce pro-mutagenic adducts believed to cause a mutation in the p53 tumor suppressor gene; a gene responsible for cell cycle regulation, initiation of DNA repair and apoptosis.[60,61] Alterations in the p53 gene, as well as B-catenin gene are also commonly reported in hepatocellular carcinoma.[62] Although each MOA may be toxin specific, there are common key events highlighted, for instance the disruption of hepatic homeostasis favoring reduced apoptotic activity paired with enhanced cell proliferation resulting in preneoplasmic foci and hyperplasia. These key events can be further advanced by a few factors including chronic inflammation, oxidative stress, NF-kβ activation, and inhibition of gap junction intercellular communication.[35]

Hepatocellular carcinoma is the most common form of primary liver cancer and originates in mature hepatocytes. Under normal "healthy" conditions, differentiated hepatocytes are quiescent and only proliferate upon hepatic injury; so much so they can replicate more than 50 times. It is paramount that current in vitro hepatic models are able to sustain long-term culture to be able to fully assess the initial toxicological insult, as well as the regenerative ability of the hepatocytes to modulate and ameliorate the adverse reactions associated with injury over time. This is particularly pertinent when evaluating the impact of a chemical or ENM exposure on genomic integrity and stability, as DNA damage can be repaired during cell division. Therefore, what may appear to induce genotoxicity following acute (24 h) exposure may no longer have the same effect following prolonged exposure. Prolonged exposure to a chemical or ENM could even result in bioaccumulation leading to metabolic saturation and a "tipping point" of toxicity.[63,64] As a result, ENMs or chemicals originally categorized as non-genotoxic following acute exposure, may actually have unforeseen, long-term adverse outcomes.

3. Human Derived Hepatocytes for In Vitro Liver Models

With the liver being an essential organ undertaking a vital role in metabolic homeostasis and the detoxification of a plethora of endogenous and exogenous substances. It is imperative that robust and physiologically relevant in vitro model systems are established to support hepatic toxicological hazard assessment following both acute and long-term exposure regimes.[19] A number of in vitro liver models have been developed and utilized to mimic the in vivo microenvironment including; human liver microsomes, human cell lines, primary human

hepatocytes (PHHs), human liver slices, and isolated perfused livers.[65,66] Regarded as a "gold standard" technique, often 2D PHHs are used to study hepatotoxicity of chemicals in vitro. However, the hepatic phenotype rapidly declines over time showing fast dedifferentiation and so they are deemed unfit for long-term exposure studies in a 2D setup. Even with additional features (e.g., an extracellular matrix [ECM] protein sandwich culture) to enhance the physiological relevance of the 2D in vitro microenvironment, PHH cultures lose their hepatic functionality after 14 days in culture, with evidence of reduced albumin and urea production.[67] Similarly, 2D systems developed with human hepatic cancer cell lines like HepaRG or HepG2, were found to exhibit reduced hepatic characteristics and metabolic activity, specifically cytochrome P450 (CYP450) enzymes (e.g., CYP1A2 and CYP3A4).[68–70] Reduced expression of phase I enzymes, such as CYP1A2 and CYP2E1, and the subsequent decline in metabolic activity has resulted in some pro-mutagenic compounds (e.g., styrene, aromatic amines, and 2,4-diaminotoulene) being difficult to detect with 2D systems alone.[71–73] Overall, 2D liver models allow for rapid low-cost, high-throughput screening of chemicals or ENMs and are suitable for the evaluation of acute toxicity. However, 2D systems generally show decreased hepatic phenotype, a reduced metabolic capability, both of which diminish further over extended culture periods, and do not emulate the complex intricacies of the 3D organ system (e.g., intracellular interactions and bile canaliculi). These factors alone have the potential to limit their predictivity for identifying hepatotoxicants. With the longevity of 2D hepatocyte models restricted to less than two weeks, they do not allow for long-term, repeated exposure scenarios to be conducted thereby missing the evolution of toxicity during chronic conditions. To study the hepatotoxic effects of ENM exposure in vitro, a robust 3D model that has demonstrated long-term stability, liver functionality, and allows for both acute, chronic, and repeated exposure regimes is required.

It is widely accepted that 3D in vitro liver models better mimic the in vivo complexities and intricate multicellular interactions than their 2D counterparts.[19,67,74,75] These features improve the longevity of in vitro hepatic models allowing for long-term and repeated exposure regimes to be investigated. In addition, these features have enabled enhanced physiological, organo-typic features like bile canaliculi, active transporter processes, and CYP450 drug-metabolizing capabilities to develop. As a result, the physiological relevance and thereby the predictivity of the models has improved. In a 3D setup, PHHs remain the "gold standard" for hepatic hazard assessment and are considered the most sensitive cell type compared to other in vitro liver cell models; HepG2, HepaRG, and Upcyte hepatocytes.[65,76,77] PHHs are considered to possess the closest representation of active hepatic metabolism (e.g., expression of phase I and phase II enzymes, transporters, and nuclear factors) similar to that found in vivo, with CYP450 activity much greater than that observed in hepatic cell lines. However, PHH models exhibit interindividual donor variation and are known to undergo significant de-differentiation during long-term culture.[77] Interlaboratory comparisons were conducted using HepG2, HepaRG, Upcyte, and PHH models to determine if the different in vitro hepatic models could correctly identify the nine drug-induced liver injury (DILI)-implicated compounds from the four non-DILI-implicated compounds. Sison-Young et al., found that PHHs positively identified eight out of the nine DILI compounds, yet this was closely followed by HepG2 cells which correctly identified six out of the nine (>66%) chemical compounds.[77] Due to the expensive, complex, and variable nature of the PHH models, demand for research and further development of hepatocyte-derived cell line models has been established.

Immortalized human cell lines are sourced from one donor and are often genetically modified or transformed in a manner that ensures they maintain an element of their original phenotype.[68] These cultures tend to be readily proliferating, amenable to subculture, resistant to de-differentiation, and are far less sensitive to environmental changes than the PHHs. These characteristics, in line with being relatively inexpensive and easily accessible, highlight cell line-derived 3D models as valuable tools for early-stage drug, chemical, and ENM toxicity screening in vitro. A number of human immortalized cell lines, like HepG2, HepaRG, and Huh7, have been successfully utilized across a variety of 3D platforms and have displayed more liver-like functionality and phenotypic consistency than their previous 2D counterparts.

More recently, both HepaRG and HepG2 cell lines appear to be the most frequently studied cell lines and have been readily used in a range of systems, from spheroids in the hanging drop format and ultralow adhesion plates through to scaffold-based (e.g., hydrogels, Matrigel) or bioreactor systems that offer more structural support to the 3D culture.[68,69,78–81] HepaRG cells originate from hepatic-differentiated, grade-one Edmonson hepatocholangiocarcinoma and have been shown to retain their bipotent hepatic progenitor-like characteristics, with a high level of differentiation and expression of typical hepatic functions, including CYP-dependent metabolism, CYP induction, and drug transporter expression.[68,82] Furthermore, HepaRG possess expression of a major organic anion transporter (MRP-2) involved in bile excretion, together with the ability to form tight junctions, both of which provide the basis for the formation of functional canicular structures allowing for the passage of bile.[68] It has been suggested that these HepaRG 3D systems exhibit enhanced metabolic functions as a result of both selective hepatocyte differentiation and accelerated maturation induced by limited cellular proliferation. Evaluation of these HepaRG cells for drug and chemical toxicity testing showed a similar response to PHH cultures when assessing the effect of acetaminophen; with a high activation of genes related to liver damage as compared to HepG2 cells, indicating this cell line could serve as a surrogate for PHHs. Gunness et al., demonstrated this further when HepaRG spheroids exposed to 0.5–80 mmol L^{-1} of acetaminophen for 24 h exhibited a similar dose-dependent response with an EC_{50} value of 2.7 mM which reflects the concentration observed in vivo. This is suggested to be directly correlated to the high levels of CYP2E1 activity producing *N*-acetyl-p-benzoquinone imine in abundance.[78] Although several HepaRG-based 3D models have been reported to better mimic in vivo-like microenvironments they do not parallel the metabolic competence and biological relevance of that found in the PHH models, lacking a stable genetic background and the ability to proliferate. Conway et al., demonstrated this with an average binucleate frequency of less

than 10.0% regardless of the concentration and exposure time to the actin polymerization inhibitor, cytochalasin B. This was deemed unsuitable in accordance with OECD Test Guideline 487 for the CBMN regulatory standard genotoxicity assessment in vitro.[83,84]

HepG2 cells, when cultured in an advanced setup may also represent a suitable alternative to PHHs for in vitro high-throughput toxicological screening. HepG2 cells have been shown to parallel if not outperform HepaRG cells in the detection of hepatotoxicants. Research has shown that HepG2 cells have a sensitivity of 80% to hepatotoxins and can correctly identify 66.7% of DILI-implicated compounds.[77,85] HepG2 cells are non-tumorigenic, epithelial-like hepatocytes derived from a hepatocellular carcinoma and can biotransform numerous xenobiotic compounds. This cell line has been well characterized and, contrary to previous literature, found to share similar gene expression profiles as PHH cultures for drug-metabolizing enzymes and transporters (DMETs) when cultured in a 3D format.[77,86] HepG2 cells are easily accessible, cost-effective, and offer limited intercellular variation whilst retaining a high level of proliferation and phenotypic stability. They are able to secrete typical liver plasma proteins including albumin, fibrinogen, and transferrin, but often lack sufficient gene expression of some essential phase I and II biotransformation enzymes, critical for certain CYP450 enzymes. Guo et al., demonstrated this when they assessed the expression of 251 DMETs including 84 phase I genes, 83 phase II genes, and 84 phase III genes in four cell lines (HepG2, Hep3B, SK-Hep1, and Huh7). In PHH, 69 out of the 84 phase I genes were detected in RNA preparations whilst only 44 phase I genes were expressed highly enough by HepG2 cells resulting in a reduced capability to catalyze oxidation, reduction, hydrolysis, cyclization, and decyclization reactions.[65] However, the transition from culturing HepG2 cells in a 2D format compared to a 3D format has enhanced their hepatic phenotype significantly. Shah et al. illustrated this enhanced metabolic capacity of HepG2 cells, when cultured in a 3D environment, with a 6-fold increase in CYP1A1 activity and a 30-fold increase in CYP1A2 compared to the equivalent 2D format.[70] Furthermore, the enhanced activity of the phase I enzymes resulted in greater sensitivity to metabolically activated genotoxicants. Whereby, no genotoxicity was observed in 2D following 24 h exposure to 5 μM of amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, yet in 3D a significant increase in fixed DNA damage was observed.[70] This highlights that it is critically important to not solely consider the building blocks of the model (i.e., the cell line and culture supplements) but also the architecture of the system (i.e., 3D cellular arrangement) and how this may enhance the primary features of the original foundations (e.g., cellular polarization, intercellular interactions, and canicular structures).

4. Three-Dimensional In Vitro Liver Model Systems

The variety of liver cell lines described in Section 3 can be adapted to suit multiple 3D platforms. Some examples of such advanced culture systems include the formation of stem-cell derived hepatic organoids, hepatic spheroids, bioreactor systems, microchips (e.g., organ-on-a-chip), bioprinted organ systems, and microfluidic flow models. All of which improve the longevity and differentiation of the cell lines into enhanced, fully functional hepatocytes within a more physiologically relevant setup. However, the majority of these novel approaches lack a robust, economical, and simple design. They often involve laborious assembly, limited accessibility to specific resources necessary for model construction, demonstrate increased variation, and require expensive equipment and skilled expertise. These challenges represent barriers for the use of such 3D model systems in high-throughput and/or screening approaches to facilitate predictive toxicology, and many of these models are not able to support genotoxicity assessment.[69]

One method that appears to overcome many of the challenges faced in 3D model design for hepatocyte systems is the development of liver spheroids, also known as microtissues. Hepatic 3D spheroid models are generated when monodispersed cells self-assemble into compact spherical structures. They are used frequently in vitro hepatic toxicology as the technique is simple by design, highly adaptable, and is shown to recapitulate the liver microenvironment well.[76,87] One simple technique to develop 3D liver spheroids is through the use of gravity in the hanging drop method as illustrated in **Figure 3**, or the use of ultralow attachment plates which are a less laborious, but more expensive alternative. Spheroids produced via these techniques often form extensive intracellular interactions and produce their own ECM. Another approach to generate 3D structures in culture is by plating cells within an ECM using a scaffold, such as Matrigel.[88] With this scaffold-based method, multiple spheroids can be generated within one well, but they often vary more in size, shape, and number compared to the scaffold-free methods. However, a low-acyl gellan gum functional polymer matrix has been found to produce uniform spheres of 115.5 ± 1.7 μm in diameter, which can also be used with human induced pluripotent stem cells.[89] In addition, for some cell types including HepG2 cells, a scaffold can help to improve the hepatic phenotype and arrest proliferation, thereby enabling long-term stability.[90] Nonetheless, using a scaffold can add additional complexity to harvesting the cells for biochemical endpoint analysis as well as hinder the uptake and penetrance of ENMs in particular when compared to scaffold-free methods.

The versatility of these basic 3D model systems offers the potential for further advancements to emulate true in vivo conditions. Examples of this have been seen in the introduction of fluidic-based systems to recapitulate blood flow,[91,92] or the addition of non-parenchymal cell types to mimic the complex interplay of immunity, cell signaling pathways, and feedback loops.[32,93] Yet, it is worth noting that the greater human resemblance and complexity the in vitro models possess, often makes it more difficult to culture, manipulate, and apply in a hazard characterization setting. Furthermore, these modifications do not fully resolve a major limitation of the current in vitro 3D systems; the lack of complex vascular structures crucial to efficient oxygenation, transport of nutrients, and waste removal from hepatic tissues, which occur in vivo. In an attempt to counteract this, cells grown in 3D culture perform these functions by diffusion or zonation alone. As a result, the

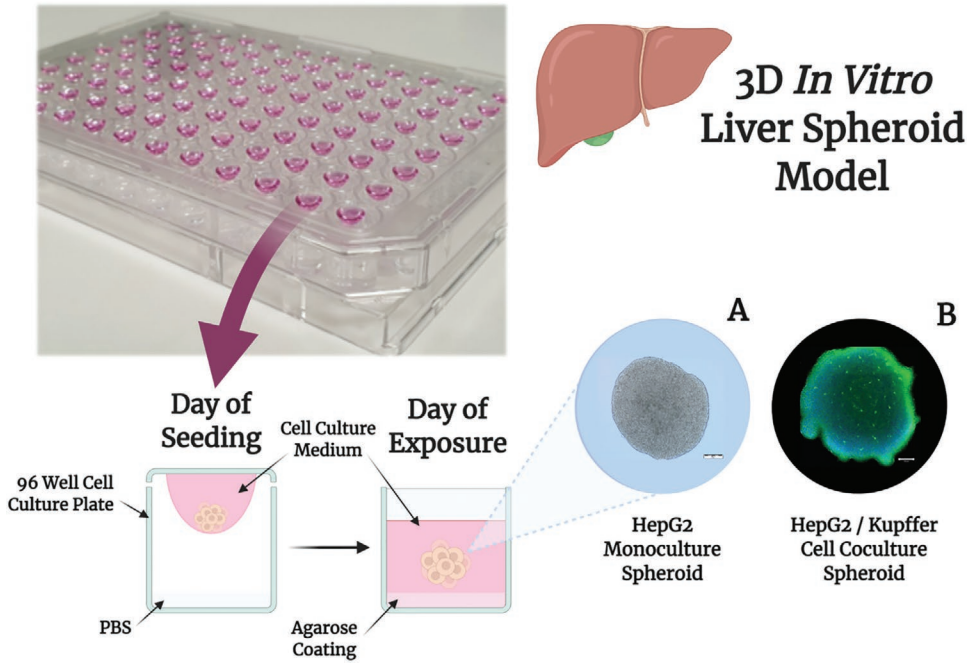

Figure 3. Illustration of a 3D in vitro liver spheroid model developed by Llewellyn et al., 2020, and formed via the hanging drop technique prior to transfer into the well plate for ENM exposure. Microscopy images of A) HepG2 monoculture spheroid and B) HepG2/Human Kupffer cell coculture spheroid taken 4 days after seeding (i.e., the day of ENM or chemical exposure) using a 10× objective with a light microscope and fluorescent confocal microscope, respectively. Green fluorescence represents the CD68 (ab222914, Abcam, UK) positive staining for the human Kupffer cells, whilst the blue fluorescence signifies the DAPI nuclear staining. The scale bars represent 100 µm. Created with BioRender.com.

longevity of these in vitro 3D model systems is often limited by cell viability as the restricted oxygen and nutrient diffusion in conjunction with an accumulation of waste at the core of the microtissue, as shown in **Figure 4**, causes a necrotic core to develop. The limit of this diffusion is thought to be ≈100–150 µm of tissue.[94,95] Based on this, a number of in vitro 3D models have been developed to be much smaller in order to limit cellular proliferation, the progression of necrosis, and extend the longevity of the cultures. Extending the longevity of the 3D model systems is an important factor when considering these in vitro liver models for use in ENM toxicity testing and hazard assessment. The continuous inclusion of ENMs into everyday applications assures that humans will be exposed to low doses of these materials on a regular, repeated basis over long durations of time. Therefore, there is a greater need for in vitro models to remain viable over extended periods of culture. A feature that a number of 3D liver models do not have without reducing the proliferative capacity of the model or increasing the complexity and subsequent production costs of that model.

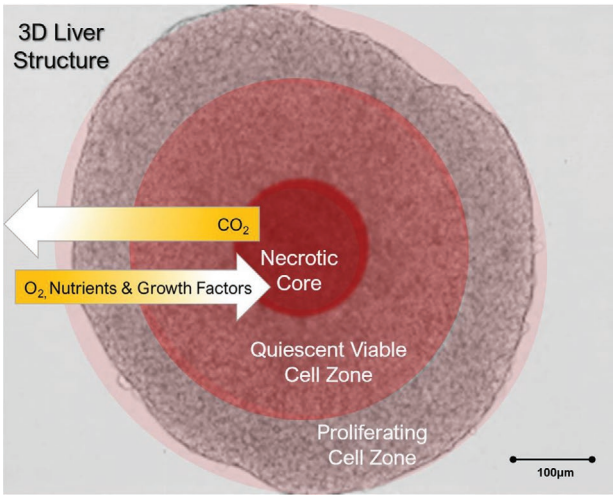

Figure 4. Diagrammatic representation of the nutrient gradient exhibited within 3D liver structure. Cellular zonation illustrated by the darkening shades of red toward the center of the 3D structure, indicating a reduction in cell viability as a result of reduced oxygen (O_2), nutrients, and growth factors and elevated carbon dioxide (CO_2) concentrations indicated by the graduated yellow arrows.

5. In Vitro Liver Model Systems for Engineered Nanomaterial Hazard Assessment

Some of the 3D liver in vitro systems described in Section 4 have already been applied for the evaluation of ENM-induced hepatotoxicity as shown in **Table 1**. However, many of the advanced 3D test systems have been designed on the basis of testing chemicals alone and do not take into consideration the challenges associated with testing ENMs. For example, a particular challenge is the variable sedimentation and diffusion rates associated with different ENMs (e.g., size, shape, and density) and variability based on the individual test system (e.g., exposure medium, scaffold material, and construction variabilities in establishing 3D models). This often results in an unequal distribution of ENMs across the test system or on

Table 1. 2D, 3D, and advanced in vitro liver models utilized for ENM toxicity screening.

	In vitro model	Suitability for ENM toxicity screening		Reference ENM studies	Cell type used	Exposure time	ENMs tested
		Pros	Cons				
2D	Monolayer	+ high-throughput + simple	– short-term only – monoculture	(Yang et al., 2019)	HL-7702	Single (24 h)	Silica NPs
				(Lorscheidt et al., 2019)	HepG2	Single (24 h)	SiO_2
				(Chen et al., 2019)	HepG2	Single (24 h)	ZnO
				(Cornu et al., 2018)	PHH, PRH	Repeated (until 3 days)	PLGA, Silica NPs
				(Brown et al., 2019)	mESC reporter, HepG2, Caco-2	Single (24 h)	TiO_2, DQ12, carbon
				(Gao et al., 2020)	hiPSC-HLCs	Single (24 h)	Ag
	PHH sandwich	+ bile canaliculi network + high-throughput	– short-term only – possible hindrance by overlay – monoculture				
	Micro-patterned co-culture	+ long-term + high-throughput + multi cell type	– lacks 3D cell-cell structure				
3D	Spheroids						
	Hanging-drop	+ long-term + equal spheroid size + multi cell type	– More labor intensive – One spheroid/well	(Fledderman et al., 2019)	HepG2	Single (24h) Repeated (until 7 days)	SiO_2
				(Conway et al., 2020)	HepG2, HepaRG	Repeated (until 5 days)	ZnO
				(Elje et al., 2020)	HepG2	Single (24h)	TiO_2, Ag, ZnO
	Ultra-low attachment plates	+ long-term + high-throughput + multi cell type + equal spheroid size	– One spheroid/well	(Kermanizadeh et al., 2019)	PHH, PHH-non-parenchymal cells co-culture	Single (24h) Repeated (until 7 days)	Ag, MWCNT, TiO2, ZnO
				(Senyavina et al., 2016)	HepaRG	Single (6h, 24h)	Ag
	Micromold	+ long-term + multiple spheroids + multi cell type + equal spheroid size	– More labor intensive				
	Bioreactors	+ long-term + large scale	– heterogenous spheroid size – low-throughput				
	Scaffold-based	+ long-term + multiple spheroids + high-throughput + multi cell type	– possible hindrance by scaffold for ENM exposure – heterogenous spheroid size	(Dubiak-Szepietowska et al., 2016)	HepG2	Single (24h, 72h)	Ag, SiO_2, ZnO
				(Lee et al., 2009)	HepG2	Single (until 24h)	CTAB-Au, citrate-Au, CdTe
	Liver organoids	+ self-organizing + expansion	– short-term only – low-throughput				
	Liver-on-a-chip	+ long-term + microfluidics + zonation possibilities + multi cell type	– low-throughput	(Li et al., 2019)	PRH	Repeated (3 and 7 days)	SPION
				(Esch et al., 2014)	HepG2-C3A co-culture	Single (24h)	Carboxylated polystyrene NPs
	Bioprinted	+ 3D architecture	– low-throughput				

the cell surface of the 3D liver model, which has the potential to cause dosimetry inaccuracies and uncertainty concerning the actual ENM concentrations applied to the culture. This limitation can be overcome theoretically using the in vitro sedimentation, diffusion, and dosimetry model, or its more recent derivatives (e.g., ISD3) often referred to with ENM suspension exposures.[96,97] A further complication with ENM hazard assessment, is the changeable nature in the physico–chemical characteristics of the materials themselves alongside their tendency to aggregate or agglomerate, as this makes predicting biological interactions and cellular uptake difficult. With these challenges in mind, it is not surprising that, unlike chemicals, ENMs do not translocate and permeate the inner cell layers of the 3D structures as efficiently. Albeit, not without its limitations, technological advancements in in vitro 3D model ENM hazard assessment techniques provide potential alternatives to reduce the reliance on animal-based testing methods.

Dubiak-Szepietowska et al. showed the applicability of 3D HepG2 cells for ENM toxicity studies. Here, HepG2 cells were plated in three different hydrogels (Matrigel, collagen type 1, and gelatin) resulting in the formation of 3D spheroids with improved hepatic phenotype and function, as demonstrated by an increase in albumin and urea production after 10 days of culture compared to the 2D system.[88] However, these spheroids displayed a decreased sensitivity toward ENM exposures for both 24 and 72 h compared to conventional 2D culture. This could be explained by improved cell-to-cell interactions and intercellular signaling in 3D culture, which not only encourages the cells to differentiate into more complex structures but alters the signaling (and possibly repair) activities in a cell. More importantly however, the hindered diffusion of ENMs in the hydrogels are likely to result in lower cellular ENM uptake during the acute phase.[88] Hence, the use of 3D models formed with scaffolds or matrices may not be suitable to study the effects of ENM exposure.

Nonetheless, 3D human liver microtissue models are shown to identify adverse reactions after repeated long-term exposure to a range of ENMs.[74,93] A study by Kermanizadeh et al. (2014) showed that scaffold-free primary human 3D liver microtissues, consisting of both PHHs and non-parenchymal cells, were able to identify hepatotoxic effects of ENMs during a 15-day, repeated exposure regime. Here, predominantly, cytotoxicity was seen after prolonged or repeated exposure scenarios and not with single exposures alone, highlighting the importance of having a long-term stable in vitro liver model to recognize ENM-induced hepatotoxicity. A more recent study, by Kermanizadeh et al. (2019), evaluated the recovery capabilities of liver spheroid microtissues after 7- or 14-day repeated ENM exposure to zinc oxide (ZnO), TiO_2, and CeO_2 and the positive control quartz containing ≈87% crystalline silica (DQ12). During the recovery periods, a reduction in cytokine production was observed, suggesting the microtissues could recover and emulate an element of liver regeneration as found in vivo. Interestingly, darkfield imaging highlighted that the ENMs could translocate through the outer surface cell layers of the spheroid and even penetrate the core, providing evidence to support that ENM exposure on 3D model systems can be comprehensive.[74] However, after two weeks of culture a decline in the viability of the untreated control suggests the 3D model system was not as stable as first thought and should be used no longer than 14 days for long-term ENM exposure screening.

Although this study highlights the suitability of this PHH 3D liver model for longer-term ENM hazard assessment, Llewellyn et al. also demonstrated that HepG2 cells could be cultured in a hanging drop format for up to 14 days without the need for specialized equipment or scaffolds.[80] The liver spheroids were ≈500 µm in diameter and able to support both acute (24 h) and longer-term (5 days) ENM exposure regimes without a significant reduction in cell viability in the untreated controls. Furthermore, HepG2 spheroids seeded at 4000 cells per spheroid, were found to retain sufficient proliferative capabilities to be able to support the CBMN assay for genotoxicity assessment following both chemical (aflatoxin B1) and ENM (TiO_2 and Ag) exposure;[80,83] a feature that other in vitro 3D hepatocyte models are currently unable to offer. Interestingly, another HepG2-based spheroid model was utilized to compare the cytotoxicity and genotoxicity using the comet assay, of Ag, ZnO, and TiO_2 ENMs following acute 24 h exposure.[98] In both 2D and 3D cultures the relative cell viability decreased in a concentration-dependent manner after exposure to Ag and ZnO ENMs, whilst TiO_2 had no effect. For ZnO ENMs, the calculated EC_{50} values were in the same range for 2D and 3D cultures: 10.1 and 16.2 µg cm^{-2}, respectively. Yet, the induced cytotoxicity of Ag ENMs was higher in 2D cultures compared to 3D cultures, with EC_{50} values of 3.8 and >30.0 µg cm^{-2}, respectively.[98] This could be attributed to the high levels of agglomeration observed with the increased hydrodynamic diameter of Ag ENMs from 37.3 ± 0.04 to 508.8 ± 29.5 nm further reducing the penetrative capabilities of the Ag ENMs into the compact, 3D spheroid structure.

Despite the advantages of using 3D models, their compact intricate structure can result in the uneven distribution and hindered penetration of ENM across the 3D structures.[99–102] A recent study by Fledderman et al. (2019) showed that SiO_2 nanoparticles could only penetrate 20 µm, corresponding to three cell layers, into the HepG2 spheroids created via the hanging drop method. In concordance, a study by Huang et al. demonstrated that Au nanoparticles showed size dependent penetration into tumor tissue or spheroids, where nanoparticles of 15 nm or larger were unable to penetrate the tissue.[101] As highlighted by these two studies and the aforementioned PHH study undertaken by Kermanizadeh et al. (2019), the translocation capabilities and localization of ENMs can vary considerably from model to model and so should be characterized for each model independently to ensure an accurate representation of ENM distribution is carried out as seen in vivo.

Whilst screening ENM cytotoxicity using in vitro models has been performed routinely, not many have been able to adapt and utilize the 3D liver models for ENM associated genotoxicity. Most ENM associated hepatic genotoxicity studies have been assessed in vivo or with 2D hepatic, monoculture systems in vitro. When performing a literature search using the terms "3D in vitro liver model nanomaterial genotoxicity," only 9 publications were identified in PubMed, whilst "3D in vitro liver model nanomaterial toxicity" retrieved 19 relevant publications and "3D in vitro liver model toxicity" returned 189 publications. This highlights the novelty of 3D in vitro liver models being utilized for ENM genotoxicity hazard assessment and a

clear knowledge gap for further research and development to be undertaken.

6. In Vitro Engineered Nanomaterial Associated Genotoxicity Assessment

Genotoxicity arises as a result of DNA damage induced by an exogenous agent which is subsequently fixed as permanent mutations to the genetic information within a cell. These mutations may lead to the incorrect transcription of DNA to mRNA required for protein translation. When a mutation alters the genetic coding for a protein, it can affect the assembly and subsequent function of that protein. Erroneous alterations to proteins that play a critical role in the body can disrupt normal development or cause malignancies, and other pathogenic effects via mechanisms like uncontrolled proliferation, mitochondrial dysfunction, or defective metabolism.[103] Multiple in vivo and in vitro studies have reported that some ENMs exhibit clear cytotoxic, (pro-)inflammatory, and sometimes genotoxic effects, thus raising concerns as to the long-term implications on human health.[104,105] Exposure to ENMs can induce genotoxicity via primary and/or secondary mechanisms. Primary mechanisms dominate in vitro ENM associated genotoxicity testing, whilst secondary genotoxicity is recognized as the main genotoxic mechanism in vivo.[28,29] Primary mechanisms can be classified into direct or indirect genotoxicity:

- Direct mechanisms of genotoxicity involve DNA damage caused by the direct, physical interaction of ENMs with the chromosomes or DNA molecule itself.[27,28]
- Indirect mechanisms of genotoxicity arise from ENM mediated induction of ROS/RNS or the release of toxic ions from soluble ENMs (e.g., zero-valent metals, like silver) that can interfere with DNA complexes or cell cycle proteins (e.g., spindle apparatus and centrioles) that can hinder cellular replication, DNA repair, and division.[27,28]

Genotoxicity is extensively linked to elevated ROS and an imbalance in oxidative homeostasis following ENM exposure. ROS can interact directly with DNA, disrupt DNA complexes, and cause DNA strand breaks, chromosomal aberrations, and alterations; all of which can induce mutations.[5,48,51,63] Secondary genotoxicity arises in vivo as a result of chronic inflammation caused by the recruitment and activation of immune cells (e.g., macrophages and neutrophils). This cascade results in the continued release of inflammatory mediators and excessive ROS produced during phagocytosis, both of which subsequently induce DNA damage in the surrounding epithelial tissue.[27,106]

ENM associated genotoxicity can differ between in vitro and in vivo settings, as the latter is reliant on biokinetic patterns and often involves interaction between multiple cell types. However, DNA damage and associated genotoxicity can be assessed in vitro using a range of assays that test particular biochemical endpoints or target specific DNA damage mechanisms, allowing for a more controlled and targeted approach. Magdolenova et al. found that only 2.58% of articles on "NP toxicity" describe genotoxicity studies; of the 112 articles found, 94 were in vitro-based genotoxicity studies whilst 22 were in vivo studies.[106] The comet assay, CBMN assay, y-H2AX, Green Fluorescent Protein (GFP)-gene reporters, and transcriptomic screening are a few examples of common techniques utilized to assess gene activation/deactivation, DNA damage, and genotoxicity in vitro. The in vitro mammalian cell gene mutation tests using the HPRT and XPRT Genes (TG476) and the in vitro mammalian cell micronucleus test (TG487) have undergone rigorous validation and have recognized OECD test guidelines. As described in **Table 2**, not all of the genotoxicity assays available are acceptable for regulatory purposes and are, therefore, only deemed suitable for research purposes in order to provide an insight into the underlying mechanisms behind ENM associated genotoxicity. Table 1 further highlights the benefits and limitations to the current assays available to assess DNA damage and genotoxicity in vitro that have the potential to be adapted for use with 3D hepatic model systems.

7. Adapting Three-Dimensional In Vitro Liver Models to Support Genotoxicity Assessment

Genotoxicity can be assessed using a number of techniques, as highlighted in Table 2 of Section 6. Each technique has specific applications and genotoxicity targeted endpoints (e.g., chromosome aberrations or fixed DNA damage foci), but not many have been adapted for use with 3D in vitro liver models, let alone been tailored to support ENM exposures, as shown by the limited publications in the scientific literature to date. Of the genotoxicity assessments performed, there are principally three techniques which have been successfully adapted to support ENM associated genotoxicity in 3D liver models: the CBMN assay, the comet assay, and bacterial artificial chromosome (BAC)–GFP reporter cell line systems.

The CBMN assay is a reliable and multifaceted technique that measures gross chromosomal damage illustrated by the frequency of micronuclei present.[107] It is a "gold standard" technique for assessing in vitro DNA damage and genotoxicity and is described by the OECD Test Guideline 487.[84,108] The CBMN assay can readily detect both aneugenic and clastogenic acting compounds alluding to potential DNA damage mechanisms. Aneugenic compounds result in the loss or addition of whole chromosomes, whilst clastogenic compounds induce gene mutations and structural aberrations, including fragmentation and rearrangement of a chromosome.[109] Furthermore, the cytostatic status of the cell can be determined using the ratio of mononucleated, binucleated, and multinucleated cells to calculate the cytokinesis-block proliferation index.[84,110,111] Although a popular and reliable method for assessing genotoxicity, the CBMN assay is rarely used with 3D in vitro hepatic models as many are static models based on primary or differentiated hepatocytes (e.g., HepaRG) which do not have the capability to actively proliferate. As fixed DNA damage is only visible after the cells have undergone one cell cycle (i.e., undergone division), with a low binucleate frequency, the CBMN assay cannot be used to accurately predict genotoxicity. Conway et al. highlighted this when assessing

Table 2. Current, available assays used to assess DNA damage and genotoxicity in vitro that have the potential to be adapted for use with 3D hepatic model systems.

In Vitro Test	Description of Assay	Genotoxicity Endpoints Assessed	Advantages	Disadvantages	Adapted to support 3D Liver Cultures	Regulatory Approval
Micronucleus Assay	Mutagenicity test system used for the detection of small membrane-bound DNA fragments, known as micronuclei, originating from the loss of a whole chromosome or fragments of a chromosome (lacking a centromere). Number of micronuclei visible in the cytoplasm of interphase cells is scored. (OECD, 2016)	Fixed DNA Damage; Clastogenicity/ Aneugenicity; Cytostasis	Multiple Endpoints Assessed; Allows for DNA repair; Evaluation and Quantification of both structural and numerical chromosomal damage; High Sensitivity when coupled with Fluorescent In Situ Hybridization; Supports both short and long-term exposure schemes	Time Consuming; Experienced scoring expertise required	Yes	OECD Approved Test Guideline for Assessing Genotoxicity In Vitro – Guideline 487 (OECD, 2016)
Chromosome Aberration Assay	In vitro test system used to identify substances that cause structural chromosome aberrations, including changes to chromosome number and/or chromosomal deletions, inversions and translocations. (OECD, 2016)	Fixed DNA Damage; Chromosome Mutations including Numerical and Structural Aberrations; Clastogenicity/ Aneugenicity	Accurate Identification and Discrimination of Chromotid and Chromosome type Aberrations; Possible co-detection of Mitotic Indices	Laborious; Requires Highly Skilled Personnel; Cost Intensive; Scoring Subjectivity	No	OECD Approved Test Guideline for Assessing Genotoxicity In Vitro – Guideline 473 (OECD, 2016)
Point Mutation Assays (e.g., Mouse Lymphoma Assay and HPRT/XPRT Gene Mutation Assay)	Gene mutation tests identify substances that induce point mutations on the X chromosome, which subsequently inactivates or modifies the function of the gene product via base pair substitutions or frameshift mutations. Evidence of this mutation can be seen in the absence of the functional gene product (protein) that affects the growth of mutant cell colonies in selective media. (OECD, 2015)	Fixed DNA Damage; Point (Gene) Mutation; Clastogenicity	Detects Point and Chromosomal mutation; Adapted for High-throughput screening	Laborious; Time Consuming Assay (Duration: 6 Weeks); Open to Human Subjectivity in Scoring Procedure; Only specific (male) cell types are suitable for detection of gene mutations	No	OECD Approved Test Guideline for Assessing Genotoxicity In Vitro – Guideline 490/476 (OECD, 2016)
Comet Assay	Alkaline (>pH 13) single-cell gel electrophoresis based assay used to identify substances that induce DNA damage, including both single and double stranded breaks in eukaryotic cells.	DNA Strand Breaks; Alkali Labile Sites; Oxidized and Alkylated Base Regions	Detects DNA damage; Fast; Cost Effective; High-throughput screening	Does not measure fixed DNA damage lesions as the damage it detects has the potential to be repaired; High variability due to lack of standardized protocol	Yes	There is no standardized method for the in vitro comet assay, but there is an OECD Approved Test Guideline for the In Vivo version of the Alkaline Comet Assay only – Guideline 489 (OECD, 2016)
Transcriptomics	Techniques, using microarray, qRT-PCR or RNA-seq, analyze the quantity of messenger (m)RNA molecules present, which reflects the genes that are being actively expressed as protein products at a given time.	Upregulation and Downregulation of Gene Transcription	Entire Genome assessed; Elucidation of DNA damage mechanisms; High-throughput screening	Expensive; Time Point sensitive	Yes, albeit limited by RNA yield from 3D culture system	No
GFP-Reporter Systems	These systems are based on a Green fluorescent protein (GFP) hybridized gene reporter used to identify the expression of specifically targeted genes (e.g., p53, γ-H2AX), which fluoresce green if the gene is actively expressed in individual eukaryotic cells.	Gene Transfer and Expression	High-throughput; Can be coupled with Live Imaging	Requires Transgenic Cell Lines; Often cultured with a Scaffold/Matrice limiting ENM Applicability	Yes	No

the suitability of HepaRG and HepG2 cell lines to support the CBMN assay, whereby HepaRG, regardless of cytochalasin B concentration and exposure time, only yields a binucleate frequency of <10% whilst HepG2 exhibited a binucleate frequency of >30%.[70,73,83] As a result, DNA damage and genotoxicity assessment are usually performed using alternative methods such as the comet assay or integrated GFP-reporters. However, recently the CBMN assay has been successfully used to detect DNA damage and genotoxicity in 3D HepG2 hanging drop spheroids following acute exposure to aflatoxin B1 and benzopyrene.[70] This research further highlighted the importance of enhanced metabolic competence in 3D model systems relative to 2D models when assessing toxicological outcomes associated with compounds which require metabolic activation. Even when restricted by the necrotic core, the proliferative capacity of the HepG2 cells on the outer surface of the spheroids is still able to support the CBMN assay.[70,80] Furthermore, the micronucleus assay was adapted and used to successfully support both acute (1 day) and long-term (5 days) genotoxicity hazard assessment of 10 μg mL^{-1} of TiO_2 and Ag ENMs.[80] Careful consideration must be taken when using the "cytokinesis-block" version of this assay for long-term or repeated exposure regimes though, as any DNA damage induced within the first few cell cycles is distributed across the mononucleated cell population, as opposed to being retained and scored within the binucleated cells. This could lead to false negatives, as the DNA damage accumulated over the period of chronic exposure can be masked. As a result, Llewellyn et al. suggested that the mononuclear version of the micronucleus assay may be more suitable for long-term or repeated exposure regimes. The micronucleus assay is a valuable tool when assessing the genotoxic effects associated with both acute and long-term ENM exposure, as to the best of our knowledge, there has been no reported interaction between the assay and ENMs.

The comet assay is commonly used for assessing DNA strand breaks and oxidized or alkylated base lesions following ENM exposure in order to provide an indication of the mutagenic and carcinogenic potential of the ENM under evaluation.[76] The comet assay can assess a few hundred to several thousand strand breaks per eukaryotic cell; a biologically relevant sensitivity range which can detect DNA damage extending from low, endogenous levels to high, almost lethal levels of damage. Previous work, using PHH models have found that exposure to ZnO, Ag, TiO_2, and multiwalled carbon nanotubes (MWCNT) induced DNA damage at varying exposure concentrations (0.5–8.0 μg mL^{-1} of ZnO and Ag, and 16–250 μg mL^{-1} of TiO_2 and MWCNT), with ZnO and Ag exhibiting the greatest DNA damaging potential and subsequent repeated ENM exposure elevating the DNA damage levels significantly.[93] Similarly, Elje et al. found that ZnO and Ag ENMs, which both display a tendency to dissociate into ions, were found to exhibit a concentration-dependent increase (0–75 μg cm^{-2}) in DNA strand breaks following acute, 24 h exposure in both 2D and 3D HepG2 cultures. However, no effect of DNA damage was observed after exposure to TiO_2 ENMs in either 2D or 3D HepG2 cultures.[76] Whilst these studies highlight that the PHH and HepG2 3D models predict a similar ENM genotoxicity outcome, they also show that the comet assay can be applied with multiple 3D models contributing to its popularity as a method for assessing ENM associated genotoxicity in vitro. Unlike the CBMN assay mentioned above, the comet assay is not restricted by cells that do not proliferate, in fact, it is a useful tool to circumvent this limitation. The main drawback of this assay is that whilst it highlights the potential of an exogenous agent to cause DNA damage, it does not take into account the ability of the cell to undergo DNA damage repair. Thus, some of the lesions detected by the comet assay may be transient, which may result in misleading positive results. To date, there are no standardized standard operating procedures or regulatory test guidelines for the in vitro comet assay, so a diverse range of methods and cell test systems have been employed leading to variable results for both chemicals and ENMs.[112] With specific respect to ENM genotoxicity assessment, there are some concerns regarding the interactions between ENMs and the comet assay. Some studies have reported the presence of ENMs in the "comet head" which could give rise to misleading results.[113] However, comparisons between the in vitro comet and micronucleus assays have shown that out of a total 70 ENMs tested, 48 (69%) were reported as positive for genotoxicity consistently in both assays.[113] It is important to note, just like the aforementioned genotoxicity assays, that high cytotoxicity is a major contributor to the misleading positive rate.[112,114,115] Yet, there is no definitive consensus on acceptable cytotoxicity ranges for the in vitro comet assay specifically. The limitations with the in vitro comet assay have been illustrated by Elespuru et al., whereby following an extensive literature review, only 55% of the 22 papers identified that used the in vitro comet assay to evaluate ENM for DNA damage induction, met the acceptability criteria.[112] With specific respect to more complex 3D and coculture models, it is not always possible to discriminate among multiple cell types using the comet assay, as all cells are lysed prior to analysis. Thus, further development of the in vitro comet approach is required to ensure it is both nano-specific and can be robustly applied to the evaluation of 3D models.

Another novel method to detect genotoxicity or DNA damage induced by ENMs utilizes a BAC–GFP transformed HepG2 cell line, cultured in a 3D format.[75,90,116,117] Here, key genes in the P53-mediated DNA damage response signaling pathway, such as P53, P21, MDM2, and BTG2, are tagged with GFP using a BAC recombineering technique allowing the detection of the activation of DNA damage signaling upon exposure at single cell level when combined with confocal microscopy.[117,118] In response to double stranded breaks in the DNA induced by a genotoxic agent (e.g., chemicals and ENMs), the DNA damage response will be activated through the recruitment of ataxia telangiectasia mutated and ataxia telangiectasia, and Rad3-related protein at the DNA damage loci. Activation of checkpoint kinases 1 and 2 (CHK1 and CHK2) ensues and subsequent post-translational modification and activation of transcription factor tumor protein 53 (P53) occurs. This, in turn, leads to activation of its downstream target genes, such as P21, MDM2, and BTG2, aiming for cell cycle arrest, DNA repair, or apoptosis.[119] Therefore, these reporter genes reflect and highlight the DNA damage response pathway mediated by P53 well at different levels within the signaling cascade. Gene activation/deactivation is time sensitive, in that genes at the beginning of the pathway cascade will be activated and initiated much earlier

than those further downstream. Subsequently, it is important to consider the optimum window of opportunity for gene activity to be assessed, as those genes targeted can often reflect the exposure time required to be undertaken.

When combining these HepG2 DNA damage BAC–GFP reporters with live cell confocal microscopy, the activation dynamics of the critical DNA damage signaling genes can be monitored frequently over extended periods of time at single cell level.[90] Studies have shown that culturing these HepG2 reporter cells in Matrigel for 21 days resulted in the formation of stable spheroids with an improved hepatic phenotype. This includes increased albumin secretion, cytochrome P450 activity, phase II conjugation enzyme and transporter expression, and activation of hepatic differentiation transcription factors, such as HNF4alpha, C/EBP, and STAT1.[75,90] Furthermore, these spheroids stop proliferating after 7 to 14 days of culture and remain stable for multiple weeks allowing the testing of long-term repeated exposures.[75,90] By utilizing these DNA damage HepG2 GFP reporters in both 2D and 3D systems, Hiemstra et al. showed a dose and time-dependent activation of the DNA damage response upon exposure to genotoxic compounds emphasizing its suitability to identify genotoxicants.[90,120] Furthermore, the 3D HepG2 reporter system showed a high resemblance to chemical-induced stress responses seen in PHH transcriptomics. This DNA damage HepG2 reporter system in 3D can also be applied for ENM-induced genotoxicity over long-term exposure regimes. As demonstrated in **Figure 5**, this system allows for the detection of DNA damage following 3-day exposure to both chemicals and ENMs using 3D spheroid HepG2 BAC–GFP reporters. Here, activation of P21-GFP was seen after exposure to aflatoxin B1, a known liver carcinogen, as well as after repeated ZnO ENM exposure over 3 days. However, since these spheroids were formed using a scaffold, as previously reported, it is likely the penetration of ENMs into the spheroid, and uptake by the cells may be hindered due to the restricted motility of ENM to translocate through the Matrigel scaffold. To validate this, intracellular measurements of ENM uptake within the spheroids should be undertaken. Alternatively, these HepG2 GFP reporters could be used in a scaffold-free 3D setting to overcome hindrance of ENM-penetration by the ECM to allow for improved uptake of ENMs within the spheroids.

In conclusion, there are number of suitable in vitro genotoxicity assays available, but they do require optimization and further development to facilitate their application to 3D liver models, in order to provide a comprehensive evaluation of the genotoxicity induced post-acute and long-term ENM exposure.

8. Discussion and Future Directions

It is important to consider the suitability of the 3D in vitro hepatic models currently available for genotoxicity evaluation, given that DNA damage is an important key event in hepatocellular carcinoma etiology. Although the four AOPs related to human liver cancer development that have been submitted to the OECD AOP-wiki are still under review, they highlight the following key biochemical endpoints: disruption to the hepatic homeostatic balance in favor of cell growth, reduced apoptotic activity, increased cell proliferation, hyperplasia in several liver cell types, and clonal expansion of preneoplastic foci cells.[35]

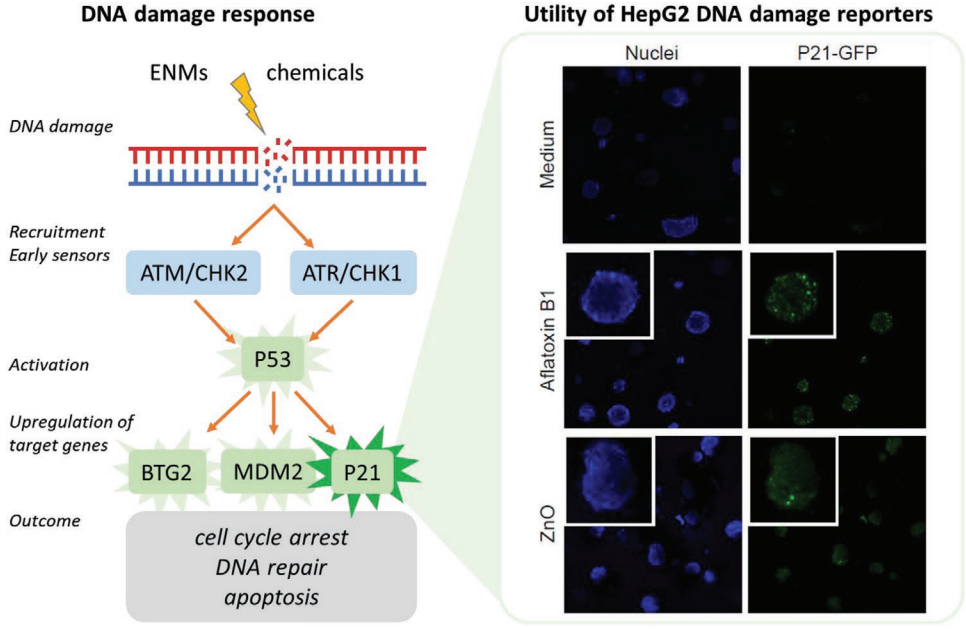

Figure 5. Mapping of the DNA damage response activation by the utility of HepG2 DNA damage reporters P21 induction upon DNA damage signaling in HepG2 cells. In the left panel, a schematic representation of the p53-mediated DNA damage response is shown. Genes in green can be monitored by the usage of specific HepG2 DNA damage reporters (P53, BTG2, MDM2, and P21). In the right panel as an example, the activation of P21-GFP HepG2 reporter upon exposure is shown. HepG2 P21-GFP spheroids grown in Matrigel were repeatedly exposed for 3 days to 5 μM aflatoxin B1 or 100 μg mL^{-1} ZnO and imaged using a confocal microscope with a at 20× objective. Cells were stained with Hoechst for nuclei visualization and propidium iodide for viability.

These parameters represent important targets that a hepatic 3D model should be able to report upon in order to provide a reliable prediction of carcinogenicity. When selecting key events to target, consideration should be given to the natural timeline of liver adverse outcomes. For example, liver cirrhosis arises as a result of prolonged exposure to hepatotoxicants causing repeated scarring of the hepatic tissue, known as liver fibrosis. Consequently, in order to supersede in vivo toxicity testing, 3D in vitro liver models must remain viable, while maintaining phenotypic functionality and stable biotransformation competence over an extended period of time. This principle has received a lot of attention in the recent scientific literature, with a number of 3D models adapted to support prolonged culture over 14 days and up to a month in some cases.[74,83,120] Not only does this development allow for more environmentally relevant long-term, repeated ENM exposures to be assessed, it also provides potential for the evolution of hepatic AOPs to be studied in a more comprehensive manner.

It is necessary to acknowledge that liver cancer progression usually evolves from DNA damage in conjunction with the loss of effective DNA damage repair mechanisms, coupled to induction of oncogenes or loss of tumor suppressor genes, such as P53.[62] A number of studies using 3D liver models have found that exposure to ENMs can induce genotoxicity in the form of DNA strand breaks and gross chromosomal damage,[74,80,98] but the majority of these studies do not take into account DNA repair capabilities or the high regeneration capacity of hepatocytes. As a result, regulatory-approved genotoxicity assays, like the CBMN assay or mutation-based assays that accommodate for DNA repair, should be the primary focus when adapting existing 3D liver models to support these endpoints. One major limitation of current 3D models, is the inability to support assays that typically detect point mutations (i.e., the mouse lymphoma assay [OECD 490] and the HPRT assay [OECD 476]). As DNA damage can be induced in multiple forms and no single assay can detect all forms of damage simultaneously, it is important to consider 3D models that are able to support an array of genotoxicity endpoints spanning point mutations, clastogenicity and aneugenicity, or other key events (e.g., cell proliferation, oxidative stress, apoptosis, and inflammation) identified in the AOP frameworks. While several of the key events have been identified to date, the molecular initiating events, particularly associated with ENM exposure, are less understood.[121] Thus, future studies should aim to identify nano-specific molecular initiating events that should form primary targets for development of novel in vitro testing strategies for hepatic disease etiology. In this manner, AOP networks could form part of a decision tree and be utilized as a foundation for establishing the most suitable in vitro test systems and biochemical assay endpoints for ENM hazard assessment.[122]

Another vital aspect of nano(geno)toxicology and the principle mechanism for genotoxicity in vivo is secondary genotoxicity, yet it remains largely overlooked within current 3D in vitro liver models.[27–29] To fully emulate the mechanisms underlying ENM associated genotoxicity, further advancement to the current 3D hepatic models needs to incorporate additional immune cell lines (e.g., macrophages and neutrophils). For example, human hepatocyte and Kupffer cell coculture models are well established in a 2D format, but immune cells are not often incorporated within a 3D approach, especially models with the capability to support genotoxicity assays. Although PHH coculture models are available to provide a more physiologically relevant alternative to in vivo toxicology models, consisting not only of human Kupffer cell macrophages but the entire nonparenchymal fraction including LSECs and stellate cells, these models are expensive and exhibit donor–donor variability in comparison to more rudimentary coculture models.[123] Furthermore, having the addition of multiple cells types means that each cell would have to be individually identified and scored independently for genotoxic events. This process could make an already labor-intensive task even more time-consuming. Based on this, future work should be focused toward developing stable, proliferative 3D coculture liver models. Resident liver macrophages have pre-eminent importance as they play a major role in the mediation of secondary genotoxicity due to their ability to phagocytose ENM and subsequently induce a sustained inflammatory response and oxidative stress.[27,106] Therefore, models which include multiple cell types, specifically Kupffer cells, in a 3D environment would be beneficial to characterize the hazards and adverse outcomes associated with ENM exposure in a more comprehensive manner.

In conclusion, adverse outcome pathways provide a useful tool for predicting specific mechanisms behind hepatotoxicity and should be taken into consideration throughout the development and refinement of in vitro test systems and relevant bioassays. This is necessary to maintain their physiological relevance and support these key events as more data are released. A major challenge has been the development of complex in vitro liver models which combine tissue-like functionality, xenobiotic metabolism competency, and retention of hepatic phenotypic characteristics over prolonged culture periods, whilst being compatible with multiparametric hazard endpoint analysis. Consequently, it may be beneficial to coalesce advantageous aspects of the existing 3D in vitro model technologies. For example, select the most phenotypically functional hepatic cell line and adapt the current culture system to mimic the setup of another model that has proven to enhance the physiological relevance, longevity, and compatibility for genotoxicity assessment. Conversely, individual models could be developed independently to address particular target endpoints, that is, PHH models for metabolism-based assessments and toxicity screening, whilst HepG2 spheroid models would be utilized for genotoxicity endpoints. Whilst multiple models are likely to be necessary to ensure predictive in vitro test systems, it is important to minimize the number of models required to support all relevant hazard characterization endpoints as in vitro approaches need to remain cost-effective and easy to implement in an industrial safety assessment setting. Although substantial advancements have been made, a need for high-throughput, robust, and physiologically relevant hepatic models capable of supporting comprehensive genotoxicity assessment following ENM exposure remains to be fully established.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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