New Approaches for Testing the (Geno)Toxic Activity of Nano-particles *In Vitro*

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Abstract:
The safety of nanomaterials, whether they are made of natural or artificial substances, represents a significant challenge because nanotechnology, as a young and up-and-coming field, is developing very quickly, while nanotoxicology and nanoecotoxicology are falling behind. Since the production, use, and consequently, the exposure of people to nanomaterials is increasing significantly, the acquisition of data on potential acute and chronic toxicity plays a crucial role. It is known that nanomaterials due to their high surface-to-volume ratio, high reactivity, and unique physical, chemical, and biological properties exhibit a greater risk of toxicity than the corresponding bulk material and that is why a comprehensive assessment of the toxicity of nanoparticles should always be done prior to their use.

We develop 3D cell models as a new *in vitro* methodological approach for nanoparticle (geno)toxicity assessment to better understand the impact nanomaterials have on environmental and human health. Currently, as a part of our ongoing study, core-shell iron nanoparticles are being examined, where the core consists of FeO, and the shell is made of Fe_3O_4. So far, *in vitro* cyto- and genotoxicity were assessed in the human hepatocellular carcinoma cell line HepG2, using the ATP assay and the comet assay, respectively, but due to ongoing genotoxicity testing and reservations about data publishing, the results will not be presented in this scientific contribution.

Keywords: 3D cell models; 2D cell models; Cytotoxicity; DNA damage; Genotoxicity; Iron-based nanoparticles

1. Introduction

Nanotechnology is a technological intersection with the nanoscale which straightforwardly links the macroscopic world of our perceptions with the nanoscopic world of individual biomolecules (Contera, 2019). It represents one of the most promising technologies of the 21st century (Bayda et al., 2019) strongly intertwined with our everyday life and society (He et al., 2019).

The prefix "nano" is of Greek origin meaning "dwarf" or something very small and depicts one thousand millionth of a meter (10−9 m) (Bayda et al., 2019) hence by the word nanomaterials we describe materials with one or more components that have at least one dimension in the range of 1 to 100 nm (Borm et al., 2006; T. Singh et al., 2017).

In the last decade, the production and use of nanomaterials have grown tremendously, and as a result, so has human exposure to these materials (Borm et al., 2006; Zhu et al., 2019). Since human exposure to nanoparticles is inevitable (Yang W, et al., 2021) much attention has been drawn to nanoparticle toxicology (Yang Y, et al., 2017) – especially to the potential acute and chronic adverse effects that nanoparticles may cause on humans (W. Yang et al., 2021) – mostly because nanomaterials due to their high surface-to-volume ratio, high reactivity, and unique physical, chemical, and biological properties (Awashra and Młynarz, 2023; Yang W,et al., 2021) exhibit a greater risk of toxicity than the corresponding bulk material (Hoet et al., 2004).

The study of nanoparticle adverse effects and toxicity is referred to as nanotoxicology (Elsaesser and Howard, 2012) and even though exposure to nanoparticles is increasing, information on their toxicological properties remains inadequate (Gornati et al., 2009). For a comprehensive assessment of the toxicity of nanoparticles, structure, and corresponding physicochemical properties need to be fully characterized because only then can the observed toxic effects be attributed to specific properties of nanoparticles in order to establish specific nanoparticle structure-activity/ toxicity functional relationships (Yang W, et al., 2021). Furthermore, to better understand the mechanisms of nanoparticle toxicity studies at the cellular and sub-cellular levels need to be done (Awashra and Młynarz, 2023).

2. *In vitro* Cell Models

2.1. *Two-dimensional (2D) cell models*

Genetic toxicity testing is an essential part of drug and material safety assessment since DNA damage can lead to genetic changes, including mutations, chromosome damage, and genomic instability that can lead to cancer development (David, 2020; Maynard et al., 2011). Current EU legislation for chemical and material safety assessment demands testing of chemical/material on two types of *in vitro* tests: (i) an Ames test (a bacterial test) and (ii) one of two mammalian cell tests – micronucleus test or chromosomal aberration test – followed by an *in vivo* animal model study (Cimino, 2006; Corvi et al., 2013). For an adequate evaluation of genotoxicity, the evaluation of three parameters is required: gene mutations, structural changes, and numerical changes in chromosomes (R et al., 2013).

In *in vitro* toxicology, the golden standard for studying absorption, distribution, metabolism, excretion, and toxicity of compounds is considered to be human primary hepatocytes, which, compared to permanent liver cell lines, better reflect the properties and phenotype of hepatocytes *in vivo*, but in practice, due to the limited availability of human liver samples, they are replaced by cell lines such as HepG2, HepaRG, Huh7, SK-Hep-1, and others. In addition, primary liver cells are difficult to maintain under *in vitro* conditions. Not only can they be grown for a short period, but they also quickly lose their cuboidal morphology and liver-specific functions during cultivation. The high price and differences between donors due to polymorphisms are also a problem (Arzumanian et al., 2021; Klingmüller et al., 2006; Sefried et al., 2018; Shulman and Nahmias, 2012; Štampar et al., 2020; Zeilinger et al., 2016).

Both human primary hepatocytes and human hepatic cell lines are normally cultured as a monolayer of cells (2D cell model) for genotoxicity testing (Laohathai and Bhamarapravati, 1985; Shulman and Nahmias, 2012) with one main difference: primary human hepatocytes cannot be cultured indefinitely as they stop dividing under *in vitro* conditions (Shulman and Nahmias, 2012). In this specific respect using human hepatic cell lines represents a good alternative for genotoxicity studies. However, one must consider that hepatic cell

lines are immortalized, cancer cells, which means that despite retaining a certain degree of the properties of primary hepatocytes, they also show similarities to tumor cells (Arzumanian et al., 2021).

While both primary hepatocytes and human hepatic cell lines as model systems have their advantages and disadvantages, both are usually used in 2D culture. 2D cell models have certain limitations for genotoxicity testing. They are indeed associated with simple and low-cost maintenance and performance of functional tests, yet they do not mimic the natural structures of tissues (Kapałczyńska et al., 2016) – 2D models lack the cell-cell and cell-extracellular matrix (ECM) signaling (Breslin and O'Driscoll, 2013; Kapałczyńska et al., 2016), which in turn leads to reduced cell differentiation and modified signaling of metabolic pathways (Aucamp et al., 2017). Furthermore, they lose the diversity of phenotype (Richter et al., 2021; von der MARK et al., 1977), and have unlimited access to the ingredients of the medium such as oxygen, nutrients, metabolites, and signal molecules (Kapałczyńska et al., 2016), forcing them into a polarization that does not reflect physiological conditions (Fontoura et al., 2020). Furthermore, the shortcomings of 2D cell models include the non-robustness of the models (Xiao et al., 2022) and misleading results (Saji et al., 2019) due to which according to ECHA (European Chemical Agency) additional *in vivo* studies need to be done ("ECHA - European Chemical Agency," n.d.; R et al., 2013). For these purposes, the European REACH regulation promotes the 3R strategy (Replacement, Reduction, Refinement), to replace and reduce the use of animals in in vivo studies, while at the same time refining test systems, obtaining more relevant results for humans (Törnqvist et al., 2014). As a result of this and the many disadvantages of 2D models, a lot of attention is being paid to the development of new alternative models that will reflect the *in vivo* conditions more accurately (Ipek et al., 2023) – and one of these are 3D cell models.

2.2. *Three dimensional (3D) cell models*

In toxicology, 3D cell models also known as spheroids are a powerful tool for studying the genotoxic effects of chemicals/materials because they better mimic *in vivo* conditions (İpek et al., 2023; Wang et al., 2021). Cells inside the spheroid comprise different cell layers (**Figure 1**) (Edmondson et al., 2014). The external layer is composed of cells displaying high proliferation rates, towards the middle resting cells can be observed, and non-dividing (necrotic) cells can be found in the core of the spheroid (Alvarez-Pérez et al., 2005; Edmondson et al., 2014; Nath and Devi, 2016). The high proliferation rate of cells in the spheroid's external layer can be explained by their easier access to oxygen and nutrients (Tredan et al., 2007). In contrast, cells within spheroids remain in a necrotic state due to the absence of oxygen (hypoxia) and nutrients (Minchinton andTannock, 2006; Tredan et al., 2007).

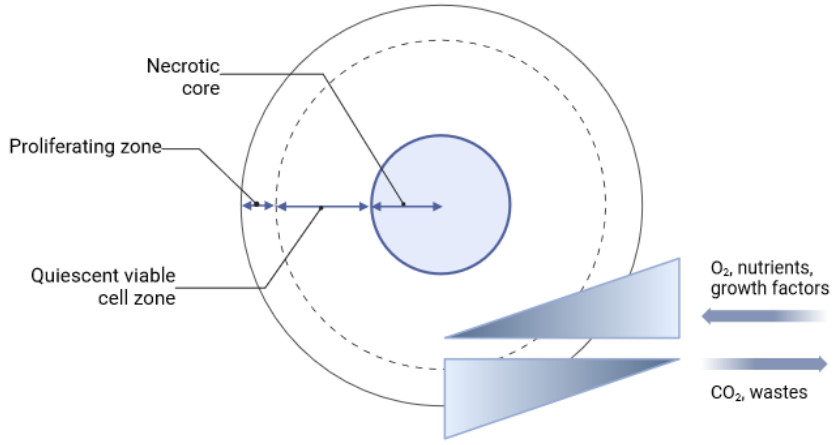

Figure 1. Structure of a spheroid (Edmondson et al., 2014; Nath and Devi, 2016). Created with BioRender.com

Apart from the 2D cell model, cells in the 3D model form better cell-to-cell connections and produce a matrix that promotes tissue-specific cell binding, direct cell-to-cell interactions, and cell-to-extracellular matrix interactions (Langhans, 2018). They also preserve their natural morphology (Costa et al., 2016) and maintain high viability for several weeks (Štampar et al., 2021). Gene and protein expression levels in spheroids better resemble levels found in cells *in vivo* (Costa et al., 2016; Langhans, 2018; Ravi et al., 2015). Therefore, by using 3D cell models, we can reduce the differences between *in vitro* and *in vivo* studies, decreasing the likelihood of needing to use animal models (Costa et al., 2016; Langhans, 2018; Ravi et al., 2015).

Despite all the advantages of 3D models, they still have certain drawbacks, including more demanding and expensive cell maintenance in culture (Costa et al., 2016; Langhans, 2018; Ravi et al., 2015), difficult replication of experiments, and more demanding interpretation of data (Kapałczyńska et al., 2016).

2.3. Spheroid formation

We are developing human hepatocellular carcinoma (HepG2) 3D cell models as a new methodological approach for nanoparticle (geno)toxicity assessment. In general, several approaches and materials can be used for culturing cells in 3D for instance, different hydrogel substrates, e.g., beads, injectable gels, moldable gels, and macroporous structures (Białkowska et al., 2020), or/and different methods such as the forced floating method (Štampar et al., 2019), pellet culture method, liquid overlay method, hanging drop method, etc. (Ryu et al., 2019). Within our department, HepG2 spheroids are routinely formed using the forced floating method (**Figure 2**), which will be described further on.

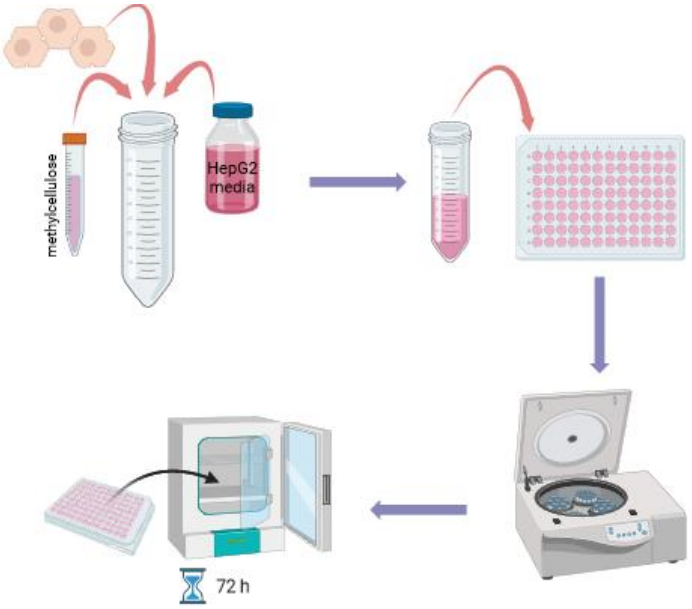

Figure 2. A simplified scheme of spheroid preparation with the forced floating method (Štampar et al., 2019). Created with BioRender.com

When preparing spheroids from HepG2 cells using the forced floating method, 96-well U-bottom low attachment microtiter plates are needed. The cell suspension is mixed with an appropriate volume of cold HepG2 medium (4 °C) – HepG2 medium is composed of MEME medium (MEME-10370-047) containing NEAA supplemented with 10% FBS, all from Gibco (Praisley®, Scotland, UK) and 2.2 g/L NaHCO3, 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin and 1 mM sodium pyruvate from Sigma-Aldrich (St. Louis, MO, USA) – and 4 % methylcellulose in a way that cell density equals 15.000 cells/mL. Then 200 µL of the mixture is pipetted into each well and plates are centrifugated for 90 minutes at 28 °C and 900 g. Due to the centrifugal force, the cells formed clusters (aggregates), which

after 72 hours of incubation (37 °C, 5 % CO_2) matured into spheroids suitable for further research (Štampar et al., 2019).

3. Methods for assessment of *In vitro* cell models

3.1. ATP Luminescent Cell Viability Assay

The ATP Luminescent Cell Viability Assay is a method for determining the number of viable cells in culture based on the quantification of the ATP present. The measurement of ATP using firefly luciferase is the most frequently applied method for estimating the number of viable cells (Riss et al., 2016) – ATP serves as an indicator of metabolically active cells because when cells lose membrane integrity, they lose the ability to synthesize ATP and endogenous ATPases rapidly deplete any remaining ATP from the cytoplasm (Riss et al., 2016). The ATP assay proved sensitive and user-friendly cell viability assay. It was reported that ATP assay is less prone to artifacts than other viability assay methods (Riss et al., 2016). Another advantage of this assay is that an incubation step with a population of viable cells is not prerequisite to convert a substrate into a colored compound (as in MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays), which also eliminates a plate handling step because cells do not need to be returned to the incubator to generate a signal (Riss et al., 2016).

3.1.1. Luminescent cell viability assay protocol

For the performance of the cell viability assay, we prepared the spheroids as described in the section 2.3 and exposed them to graded nanoparticle concentrations prepared as described in the section 3.3. After 24-hour or 96-hour exposure, we transferred 5 spheroids for each concentration from the U-bottom microtiter plate to the white opaque walled microtiter plate and added 50 µL of the reagent (CellTiter-Glo®, Promega, Madison, Wisconsin, USA) which contains a detergent to lyse the cells, ATPase inhibitors to stabilize the ATP that is released from the lysed cells, luciferin as a substrate, and the stable form of luciferase to catalyze the reaction that generates photons of light (Riss et al., 2016). Then we resuspended the mixture of reagent and cells and incubated it for 10 minutes at room temperature before measuring the luminescence signal.

3.2. Comet Assay

The comet assay, or Single-Cell Gel Electrophoresis (SCGE), is an extremely sensitive and fast quantitative in vitro method that detects DNA damage at the level of a single cell (Nickson and Parsons, 2014). The method enables the detection of single- and double-strand DNA breaks, alkali-labile sites, DNA-DNA, and DNA-protein cross-linking (Tice et al., 2000). DNA damage can be either endogenous or exogenous. Most of the endogenous DNA damage arises from the chemically active DNA engaging in hydrolytic and oxidative reactions with water and reactive oxygen species (ROS), that are naturally present within cells while exogenous DNA damage occurs when environmental, chemical, and physical agents such as UV and ionizing radiation, alkylating agents, and crosslinking agents damage the DNA (Chatterjee and Walker, 2017).

Nowadays the method represents one of the standard methods for DNA damage evaluation with applications in genotoxicity testing, human biomonitoring, molecular epidemiology, eco/genotoxicology, and basic research on DNA damage and repair (Collins, 2004; Cordelli et al., 2021).

In the SCGE method, the cells are embedded in an agarose gel, and lysed so that only the nuclei (DNA) remain in the gel. The gel is then exposed to an electric field in the electrophoresis and because the damaged DNA migrates at a different rate than non-damaged DNA using specific dyes we can observe structures, resembling a comet. The undamaged DNA in the comet structure is referred to as the "head" while the trailing damaged DNA band is referred to as the "tail". The percentage of DNA in the tail is directly proportional to the percentage of DNA damage that has occurred in a particular cell (Nickson and Parsons, 2014; Vandghanooni and Eskandani, 2011).

A simplified procedure scheme is shown in **Figure 3**.

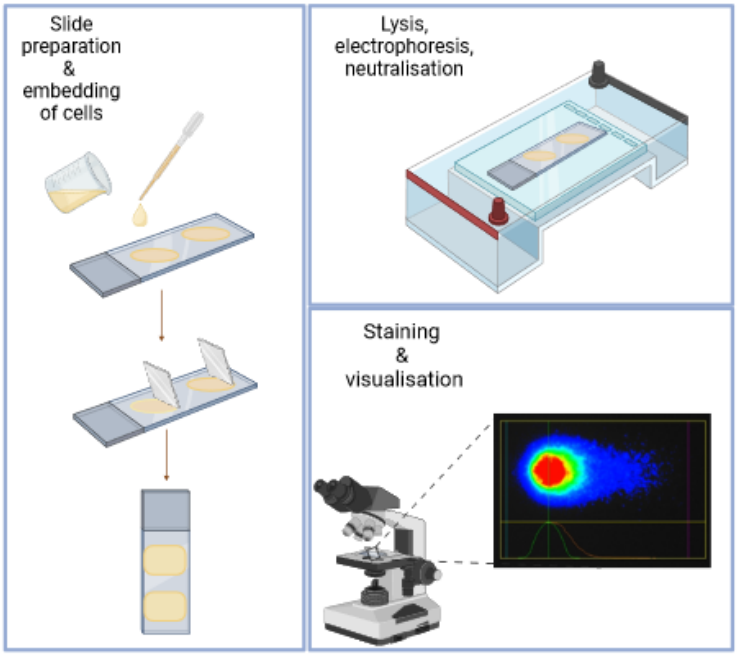

Figure 3. A simplified procedure scheme of the comet assay (Nickson and Parsons, 2014; Vandghanooni and Eskandani, 2011). Created with BioRender.com

3.2.1. Comet Assay Protocol

After the treatment (section 3.3), a suspension of viable single cells was obtained by the combination of enzymatic digestion and mechanical degradation. For each tested concentration we harvested 5 spheroids and transferred them to a 1,5 mL Eppendorf tube. Then we centrifuged them for 4 minutes at 1000 Rotations Per Minute (RPM) and discarded the media. We washed them with 1 mL 1x Phosphate Buffered Saline (PBS) and repeated the centrifugation step. After that, we discarded the PBS and added 50-100 µL of an enzymatic mixture (10x diluted collagenase (50 mg/ml) solution with serum-free medium (MEME-10370-047, Gibco, Praisley®, Scotland, UK) and TrypLE (Gibco; 12604-013, Waltham, Massachusetts, USA) in the ratio of 1:2) into the Eppendorf Safe-Lock Microtube (Eppendorf, Hamburg, Germany) with spheroids. We incubated the spheroids for 8-10 minutes at 37 °C and 5 % CO_2. After the incubation, we resuspended the spheroids and added 500 µL of HepG2 growth media with supplements to deactivate the collagenase type I and centrifuged them for 4 minutes at 1000 rpm. We discarded the media and then the comet assay was conducted according to Singh et al. (1988) with minor modifications by Štampar et al. (2019).

3.3. Nanoparticle sample preparation

So far, our work has focused on (geno)toxicity assessment of core-shell iron nanoparticles – the core consists of FeO while the shell is made of Fe_3O_4. First, we evaluated the cytotoxicity of graded concentrations of nanoparticles using the ATP Luminescent Cell Viability Assay. Nanoparticles were dispersed into cell media with the highest concentration of 80 µg/cm² and the lowest concentration of 0,2 µg/cm² for 24-hour exposure while the highest concentration for 96-hour exposure was 40 µg/cm² and the lowest concentration was 0,02 µg/cm². The concentrations were determined based on the OECD (Organisation for Economic Co-operation and Development) TG 487 guidelines (OECD, 2022) for testing manufactured nanomaterials in a way that the concentration of 40 µg/cm² equaled the concentration of 100 µg/mL. Usually, it is recommended that the top dose is restricted to 100 µg/mL or 100 µg/cm², whichever is higher, because doses higher than this are not physiologically relevant, and can result in interference with scoring due to high deposition on cells (OECD, 2022). As a positive control, 15 % DMSO

was used, since currently, there are no suitable nanoparticles that can be used as positive controls for the in vitro assays (OECD, 2022).

4. Conclusion

In general, the toxicity of nanoparticles can be assessed with several different approaches, among which, the most beneficial ones in terms of cost and time saving are the *in vitro* studies. *In vitro* studies are essential to identify biochemical and molecular mechanisms of nanoparticles' cyto- and genotoxicity and are also the first step in identifying potentially harmful effects for humans and the environment. When it comes to *in vitro* studies, 3D cell models also known as spheroids are a powerful tool for studying the genotoxic effects of chemicals/materials. Not only do they mimic *in vivo* conditions better, but their usage allows us to minimize testing on animals, thus following the 3R (Replacement, Reduction, and Refinement) principle. Furthermore, spheroids represent a promising model for nanoparticle (geno)toxicity assessment and regarding that, significant progress has already been made. However, a lot of inadequate information is available and that is why more research needs to be done to identify the most appropriate approach for assessing nanoparticle toxicity.

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