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Methods and protocols in nanotoxicology

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Methods and protocols in nanotoxicology

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Editorial: Methods and protocols in nanotoxicology

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nanotoxicology, protocols, interference, genotoxicity, reproducibility

Editorial on the Research Topic
Methods and protocols in nanotoxicology

Although the first studies on the toxicology of nano-scale materials (colloids) were carried out nearly 100 years ago, the enormous increase in the number of studies on nanomaterials only began with the euphoria triggered by the targeted manipulation of matter at the atomic level. As a consequence, major concerns have been raised about the risks behind this technology (Hoet et al., 2004; Stern and McNeil, 2008). National or international initiatives or action plans have been established in many countries (cf. the National Nanotechnology Initiative [NNI], launched in 2000 in the United States, and the European Commission's report *Nanosciences and Nanotechnologies: an action plan for Europe 2005–2009*, published in 2005). All of these initiatives contained funding programs focused on health and environmental impacts of nanomaterials. This circumstance led to a dramatic increase in the number of materials studied as well as publications on the biological safety of these materials (Figure 1). It quickly became obvious that nanomaterials pose a lot of problems when tested in biological assays. To be mentioned here are the interferences of the material with the test itself (Wörle-Knirsch et al., 2006; Kroll et al., 2012; Guadagnini et al., 2015). Furthermore, although the name is often identical (e.g., carbon nanotubes), the materials used are very different (e.g., single walled, multi-walled, short or long fibers, rigid and stiff or flexible and entangled), which makes an intensive characterization necessary in order to be able to classify the results correctly (Warheit, 2008; Crist et al., 2013). In addition, materials that have been on the market for a long time were hardly perceived as "nanomaterials" (e.g., TiO_2, SiO_2, carbon black), but these are now under discussion although registered as market products, such as TiO_2.

In view of the enormous number of publications on nanotoxicology (> 60.000 since 2000, see Figure 1), the critical questions must be addressed: why is there still so much uncertainty in the statements on possible biological effects and why are results so inconsistent? Especially the reproducibility of results is in many cases very weak, although this is not restricted to nanotoxicology (Baker, 2016). Various scientists have criticized this situation (Hirsch et al., 2011; Krug, 2014; Petersen et al., 2014). Others tried to give answers and made suggestions for better reproducibility (Petersen et al., 2020), for

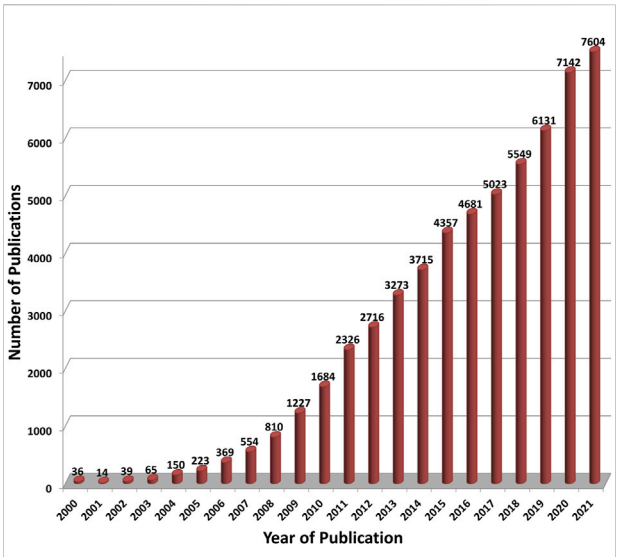

FIGURE 1
The number of publications on "Nanotoxicology" *per* year as found in the meta-database PubMed (https://pubmed.ncbi.nlm.nih.gov/). For each year from 2000 to 2021 (abscissa) publications have been searched within this database with the following search profile: "all fields" contain "nanotox*" or "fulleren* AND toxic*" or "nanotube AND toxic*" or "nanoparticle* AND toxic*" or "nanomat* AND toxic* or "nano* AND toxic*" or "graphene AND toxic" where the asterisk is a wild card.

enhancing the overall quality of studies (Fernández-Cruz et al., 2018) and "to generate inherently FAIR[1] nanosafety data to support the efficient governance and regulation of nanomaterials" (Jeliazkova et al., 2021). In this context, there are still very important demands that have not yet been adequately met, despite the major funding programs and many national and international projects. This is because there is still a lack of harmonized protocols that are accepted in the scientific community. So far, some of the OECD test guidelines for the testing of chemicals have been adapted for nanomaterials, but the numerous protocols established and standardized in European projects are mostly not used in the in-depth study of nanomaterial toxicity in many laboratories. In fact, there are several European activities (e.g., the Malta Initiative[2]) and projects which contribute to the development or adaptation of OECD test guidelines for nanomaterials (e.g., NANOHARMONY[3], Gov4Nano[4], NANORIGO[4], RiskGONE[4]). The German project DaNa has compiled a collection of standard operating procedures (SOPs) and laboratory protocols from different initiatives and published them online. In various subcategories, such as Biological Test Methods, Physico-Chemical Properties, Sample Preparation, one can download SOPs and laboratory protocols as pdf files. In addition, the DaNa team has set up a template with filling-in help for the creation of SOPs[5]. This pioneering activity is now complemented by EU projects and an SOP handbook is available at the website of the Horizon 2020 project PATROLS[6].

For this reason, project activities are launched and journal special issues like this one are published to help improve the situation. The study of nanomaterials and their potential biological effects usually starts with *in vitro* cytotoxicity assays, which may be misleading because of interferences between the tested material and the assay components, as has been shown previously (Wörle-Knirsch et al., 2006). To overcome these problems, the Alamar Blue assay was further developed so that it can be reproducibly applied even in high-throughput experiments (Longhin et al.). A second example as an alternative for viability measurement is the colony-forming efficiency assay. This viability assay has been optimized for high-throughput experiments as well and is practically interference-free as no dyes are used. Moreover, the treatment time can be prolonged up to 10 days which can be regarded as a sub-chronic assay (Runden-Pran et al.). As a next step in the *in vitro* toxicity assessment the induction of oxidative stress is an important pathway of toxicity. Most often this endpoint is analyzed by using the fluorescence dye DCF, but this assay is like other fluorescence dye-dependent assay systems error-prone (Petersen et al., 2020). Alternatively, a better and more reliable analysis can be performed *via* the expression of anti-oxidative enzymes under the control of the nuclear erythroid 2-related factor 2 (NRF2) transcription factor. A world-wide consortium has developed a reporter gene assay for the measurement of NRF2 mediated gene expression and validated it *via* intra- and interlaboratory round robins (Martin et al.). Although the variability of the intra- and inter-laboratory results is relatively low, it becomes obvious that the higher the induction of expression, the higher is the variability between the labs.

The genotoxicity potential of TiO_2 has recently been (re) evaluated but is still under discussion, and this clearly demonstrates the need for better and more reliable genotoxicity testing. Since this is the most important endpoint in the toxicological evaluation of a substance, the further development of existing assays and the establishment of new reliable tests is essential. Until now, the Comet assay has been criticized for being error-prone and providing biased results

1 FAIR: findable, accessible, interoperable, reusable data.

2 https://www.nanosafetycluster.eu/international-cooperation/the-malta-initiative/.

3 https://nanoharmony.eu/.

4 https://www.nanosafetycluster.eu/nsc-overview/nsc-structure/steering-group/.

5 https://nanopartikel.info/en/knowledge/operating-instructions/.

6 https://www.patrols-h2020.eu/publications/sops/index.php.

(Rajapakse et al., 2013; Ferraro et al., 2016). To avoid these weaknesses, an improved protocol was established that takes into account both cytotoxicity and uptake of nanoparticles by cells and establishes clear test acceptance criteria and consideration of historical controls (El Yamani et al.). The use of this approach will make the *in vitro* Comet assay much more reliable in the future. A similar study has set itself the task of adapting the existing protocol for the *in vivo* comet assay (OECD test guideline 489) so that the protocol can also be used for nanomaterials (Cardoso et al.). A comparable goal was set by another group. Here, the OECD test guideline 490 (thymidine kinase gene mutation test) was adapted for testing of nanomaterials. Also, with these changes to the existing protocol, care was taken to ensure that there are clear acceptance criteria and that the specific nanomaterial-related properties are considered (Chen et al.). It remains to be hoped that these adjustments to the existing OECD guidelines may be accepted by the scientific community and incorporated into the official protocols as soon as possible. The results of nanomaterial genotoxicity studies are often misleading as no discrimination between primary and secondary genotoxicity has been done. However, because many nanomaterials can induce oxidative stress or inflammatory processes that then indirectly lead to subsequent DNA damage, secondary DNA damage is often underestimated, and the overall genotoxicity of nanomaterials is overestimated. To better capture this shift in results, a co-culture system was established that discriminates well between primary and secondary genotoxicity (Vallabani and Karlsson). Using the example of nickel oxide nanoparticles with corresponding positive controls, it was shown that the cells used react significantly differently and human bronchial epithelial cells show exclusively secondary DNA damage. A further advantage of this protocol is the analysis of micronuclei by means of flow cytometry which reduces the possible bias. Taken together, with regard to genotoxicity, the articles in this special issue refer to various difficulties in the different steps of the individual methods when nanomaterials have to be investigated. As a kind of overview, another article in this series therefore addresses precisely these problems step by step and gives clear recommendations for avoiding them (Elespuru et al.). This article does not criticize the methodological errors of previous studies (what is wrong) but shows how the individual publication in this series addresses the critical points with positive advice to avoid these errors (what is right). The final article in this series covers a more basic aspect: establishing more realistic *in vitro* test systems. By using a microfluidic serum-free cell-on-a-chip system, it could be shown that dynamic conditions may reflect the tissue response in a more accurate way (Gupta et al.). In the future, such microfluidic systems are likely to encompass multiple cell types that can represent an entire organ, and may help to reduce animal testing, and increase the significance of *in vitro* approaches.

The methods and protocols presented in this special issue are intended to help improving transferability and reproducibility of results from different laboratories. Many potential sources of error have been identified and interferences of nanomaterials with assay systems have been demonstrated. The same applies to nanomaterials as for other chemicals: toxicological data are only useful and usable if they are confirmable by other laboratories.

Author contributions

HK and KN wrote the article.

Conflict of interest

HFK is shareholder of NanoCASE GmbH.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Development of Microfluidic, Serum-Free Bronchial Epithelial Cells-on-a-Chip to Facilitate a More Realistic *In vitro* Testing of Nanoplastics

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Most cell culture models are static, but the cellular microenvironment in the body is dynamic. Here, we established a microfluidic-based *in vitro* model of human bronchial epithelial cells in which cells are stationary, but nutrient supply is dynamic, and we used this system to evaluate cellular uptake of nanoparticles. The cells were maintained in fetal calf serum-free and bovine pituitary extract-free cell culture medium. BEAS-2B, an immortalized, non-tumorigenic human cell line, was used as a model and the cells were grown in a chip within a microfluidic device and were briefly infused with amorphous silica (SiO_2) nanoparticles or polystyrene (PS) nanoparticles of similar primary sizes but with different densities. For comparison, tests were also performed using static, multi-well cultures. Cellular uptake of the fluorescently labeled particles was investigated by flow cytometry and confocal microscopy. Exposure under dynamic culture conditions resulted in higher cellular uptake of the PS nanoparticles when compared to static conditions, while uptake of SiO_2 nanoparticles was similar in both settings. The present study has shown that it is feasible to grow human lung cells under completely animal-free conditions using a microfluidic-based device, and we have also found that cellular uptake of PS nanoparticles aka nanoplastics is highly dependent on culture conditions. Hence, traditional cell cultures may not accurately reflect the uptake of low-density particles, potentially leading to an underestimation of their cellular impact.

Keywords: alternative methods, *in vitro*, microfluidics, nanoplastics, nanotoxicology

INTRODUCTION

Experts in the field have argued that "nanotoxicology is currently at a crossroads and faces a number of obstacles and technical limitations not associated with traditional toxicology" (Hussain et al., 2015). In fact, the field of nanotoxicology still relies heavily on assays and methods developed for the testing of traditional chemicals, and the development of relevant and robust assays amenable to high-throughput screening of nanomaterials represents an important priority (Li et al., 2018; Fadeel, 2019). There is a strong consensus that faster and animal-free approaches for safety assessment of

chemicals as well as engineered nanomaterials are needed (Kohl et al., 2021). Conventional cell culture models fail to recapitulate the dynamic environment of a living system, and microfluidic cell culture systems have emerged in recent years as a promising alternative with the potential to replace or at least reduce the use of animal experiments (Bhatia and Ingber, 2014; Ingber, 2020). Huh et al. (2010) developed a mechanically active lung-on-a-chip device and were able to demonstrate that cyclic mechanical strain to simulate breathing accentuates the toxicity of silica nanoparticles (NPs). More recent developments include the design of multiorgan-on-a-chip devices in an attempt to capture the crosstalk between different cell types (Ashammakhi et al., 2020). Additionally, recent attempts have been made to grow tumor spheroids in a microfluidic device to more accurately model and determine NP uptake (Zhuang et al., 2019).

Using microfluidics-based cell culture systems, several investigators have provided evidence that NPs may display different effects under dynamic flow conditions as opposed to conventional, static cell culture conditions. For instance, Kim et al. (2011) investigated the cytotoxicity of mesoporous silica NPs towards immortalized human endothelial cells under flow conditions and found that the NPs showed higher toxicity under flow conditions when compared to static conditions. In contrast, Fede et al. (2015) evaluated the toxicity of ultrasmall gold NPs towards human umbilical vein endothelial cells (HUVEC) under static and flow conditions and found that the toxicity was reduced under flow conditions. In the latter study, uptake of NPs under flow was found to be lower than in static conditions. Using a panel of cancer cell lines, Kang et al. (2016) showed that cellular uptake of polystyrene NPs (100 nm) was higher under shear stress conditions when compared to static cell cultures. Other investigators have shown, using a panel of solid vs. hollow silica NPs of roughly the same size (350 nm), that the particle density affected cellular uptake and toxicity under flow conditions (Yazdimamaghani et al., 2018). Xu et al. (2020) developed a lung-on-a-chip model consisting of endothelial cells and epithelial cells to recapitulate the alveolar-blood barrier (to study fine particulate matter). However, the divergent outcomes of these studies suggest that important differences exist not only between static and dynamic cell culture conditions, but also depending on the cell types used and on the types of NPs.

Plastics have outpaced most man-made materials yet none of the commonly used plastics are biodegradable, and plastic debris therefore accumulates in the environment (Geyer et al., 2017). Consequently, numerous studies have addressed the environmental impact of plastic litter and microplastic fragments (for a review, see Mitrano et al., 2021). However, few studies have focused on the potential human health effects of microplastics or the nanoscale breakdown products commonly referred to as nanoplastics. Most *in vitro* toxicological studies use polystyrene (PS) NPs as model particles and, for the most part, toxicity is only observed at high concentrations or following long-term exposure, unless the NPs are endowed with a positive surface charge, as is the case for amino-functionalized PS NPs which were shown to be toxic towards a range of cell types (Anguissola et al., 2014; Mrakovcic et al., 2014; Ruenraroengsak and Tetley, 2015; Hesler et al., 2019; He et al., 2020). Notwithstanding the fact that spherical NPs may not be representative of the heterogenous features of plastic debris (Gigault et al., 2021), the question remains whether traditional cell culture models are suitable for the evaluation of nanoplastics.

Cho et al. (2011) investigated cellular uptake of gold NPs using upright and inverted cell culture configurations and found that uptake depends on the sedimentation and diffusion velocities and is independent of size, shape, density, and surface coating of the NPs. Thus, the toxicologically relevant dose should take sedimentation into account (Lison and Huaux, 2011). However, the gold NPs used in the latter study may not accurately reflect the behavior of low-density nanoplastics. In fact, Watson et al. (2016) showed in a seminal study that the toxicity of low-density NPs may be overlooked when using conventional cell culture models. The authors tested polypropylene (PP) NPs and compared conventional and inverted cell culture platforms using primary human monocyte-derived macrophages maintained in standard medium supplemented with fetal bovine serum (FBS). No toxicity was observed in the conventional set-up whereas a dose-dependent decrease in cell viability and an increase in reactive oxygen species production was observed in the inverted cell culture system. The authors argued that due to the buoyancy of the NPs, there is "essentially zero dose delivered to the cells at the bottom of the well" when administering the particles in the conventional model (Watson et al., 2016). However, not all cells can be maintained upside-down in culture, and a model in which a dynamic flow is applied using a microfluidics-based system may be a more relevant way of addressing low-density NPs such as nanoplastics. The present study seeks to develop a more realistic *in vitro* model of the human lungs based on serum-free culture of a human lung cell line using cell culture-on-a-chip microfluidic technology. We prepared a step-by-step guide for the assembly of the test platform, which can be found in the supplement accompanying this paper. Furthermore, as a proof-of-concept, we studied fluorescent silica NPs and PS NPs of similar size (45 and 50 nm) and determined cellular uptake under static vs. dynamic cell culture conditions using the BEAS-2B cell line.

MATERIALS AND METHODS

Microfluidic System and Cell Culture-on-a-Chip Model

A step wise description of the assembly of the microfluidic system is provided in the **Supplementary Box 1**. The system is comprised of five different parts, including the cell culture-on-a-chip (COC) procured from Micronit (Netherlands). The resealable top and bottom layers are of same width (15 mm) and length (45 mm) as the 0.4 mm glass middle layer. Assembly of top and bottom layers resulted in the formation of two flow chambers separated by a middle layer that contains the cell culture membrane. Hence, a cavity with a polyester (PET) membrane fixed on a glass slide, with a thickness of 12 μm

and pore size of 0.45 μm, with 1.6×10^6 pores/cm^2 density and 1.6 cm^2 surface area, separates the upper chamber (UC) and lower chamber (LC). Both upper and lower slides were spaced from the middle glass layer membrane *via* a silicone gasket (0.25 mm), resulting in a volume of 110 and 75 μL for the UC and LC, respectively, and a total volume of 185 μL for the device. The created distance from the middle layer to either top or bottom layer was 0.25 mm whereas the distance between the top layer and the membrane on middle layer cavity was 0.65 mm. The chip was mounted with a quick locking mechanism in the chip holder constructed for connecting external tubing to the chip *via* ferrules to ensure tight connections and a leak-free system. The specified NPs were added to two separate input glass bottles (50 ml) connected through the digitally operated OB1 MK3+ pressure controller by Elveflow (Elvesys, France). The glass bottles were then connected to the UC and LC compartments of the COC with polytetrafluoroethylene (Teflon) tubing (0.25 mm inner diameter, 14.5 cm length) through the Fluidic Connect Pro chip holder (Micronit). The same tubing was used on the outlets of the chip to connect with the glass collecting reservoirs. Prior to cell culture experiments, all tubing and chip parts were placed under the UV light in a laminar air flow and sterilized using 70% ethanol and the tubing was filled with medium to eliminate air bubbles. The microfluidic system was placed in an incubator at 37°C to sustain cell culture conditions.

Animal-Free Culture of Human Bronchial Epithelial Cells

The immortalized human bronchial epithelial cell line, BEAS-2B (European Collection of Cell Cultures) was cultured in PneumaCult™-Ex Plus Medium (Stemcell Technologies, United Kingdom) supplied with 50x extra supplement; hydrocortisone (Stemcell Technologies, United Kingdom) and penicillin-streptomycin solution (Gibco, Sweden) was added to the complete cell medium. It is important to note that the cell medium is free from FBS and bovine pituitary extract (BPE). Hence, the cell culture medium can be considered "animal-free" (Oredsson et al., 2019). Furthermore, BEAS-2B cells are often grown on a substrate of fibronectin, collagen, and serum albumin of bovine origin. However, we were able to maintain cells without pre-coating with extracellular matrix proteins (Supporting Information), thus avoiding the use of animal proteins. Hence, the cells were seeded in 75 cm^2 tissue culture flasks without pre-coating and expanded until 70–80% confluence for further studies under static or dynamic conditions, as described below.

Fluorescence Microscopy and Cell Viability Assessment

Cell imaging: For optical and fluorescence microscopy, cells were seeded overnight on glass coverslip placed in a 24-well plate or in the microfluidic chip. Next, cells washed with PBS and fixed with paraformaldehyde (4%). Thereafter, cells were washed and stained with CellMask™ Deep Red to visualize the plasma membrane (Thermo Fisher Scientific) and counterstained and mounted with ProLong™ Gold Antifade Mountant containing DAPI to visualize cell nuclei (Thermo Fisher Scientific) and imaged using the EVOS™ M7000 imaging system (Thermo Fisher Scientific) at 400x magnification. *Cell viability*: BEAS-2B cells were seeded at a density of 60,000 cells/cm^2 either in a 24-well plate or on the microfluidic chip. Cell supernatants were collected 24 h after seeding and LDH release was measured for cell viability assessment by using the CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega).

Preparation of Silica and Polystyrene Nanoparticles

FITC-labelled colloidal SiO_2 NPs (primary size 45 nm, density ~2.0 g/cm^3) and Dragon Green™-labelled polystyrene (PS) nanoparticles (primary size 50 nm; density ~1.06 g/cm^3) were used in the present study. Fluorescent SiO_2 NPs were prepared using a modified Stöber synthesis (Pihl et al., 2019). In brief, a fluorescent precursor was prepared by reacting fluorescein isothiocyanate (FITC) with (3-aminopropyl) trimethoxysilane. This conjugate was then condensed with tetraethyl orthosilicate (TEOS) to yield fluorescent particles in a mixture of water, ethanol and ammonia. The particles were purified and further coated with an extra layer of silica. An advantage of this approach is that the fluorophore is incorporated in the core of the NPs thus preventing leakage of the dye while preserving the surface chemistry of the NPs. The FITC-SiO_2 NPs were used for experiments after dilution at the desired concentrations in cell culture medium. The PS NPs were obtained from Bangs Laboratories, Inc. (USA). According to the manufacturer, the latter NPs were produced by embedding the fluorescent dye internally leaving the surface groups of the NPs unaltered. However, the colloidal suspension (1% solid or 10 mg/ml) of PS NPs contained surfactant (<0.1%) as well as biocide (NaN_3) (<0.09%). This may obviously skew the results (Heinlaan et al., 2020). Therefore, the NPs were dialyzed for 3 days using the 10 kDa gamma-irradiated Slide-A-Layer™ dialysis cassettes (ThermoScientific). Water was changed every day during dialysis for three consecutive days. The SiO_2 NP and PS NP stock solutions were dispersed in cell culture medium at 10 μg/ml and gently vortexed for 30 s prior to the experiments with BEAS-2B cells. For characterization, NPs were collected from both inlet and outlet reservoirs connected to the microfluidics system. After 1 h of exposure, the samples were collected from inlet and outlet reservoirs and characterization was performed. Hydrodynamic diameter and ζ-potential measurements were performed as described previously (Bhattacharya et al., 2017) using the Zetasizer Nano ZS90 (Malvern, UK).

Exposure of Cells Under Dynamic and Static Conditions

A step wise description of the assembly of the cell culture-on-a-chip (COC) is shown in the **Supplementary Box 2**. Briefly, BEAS-2B cells

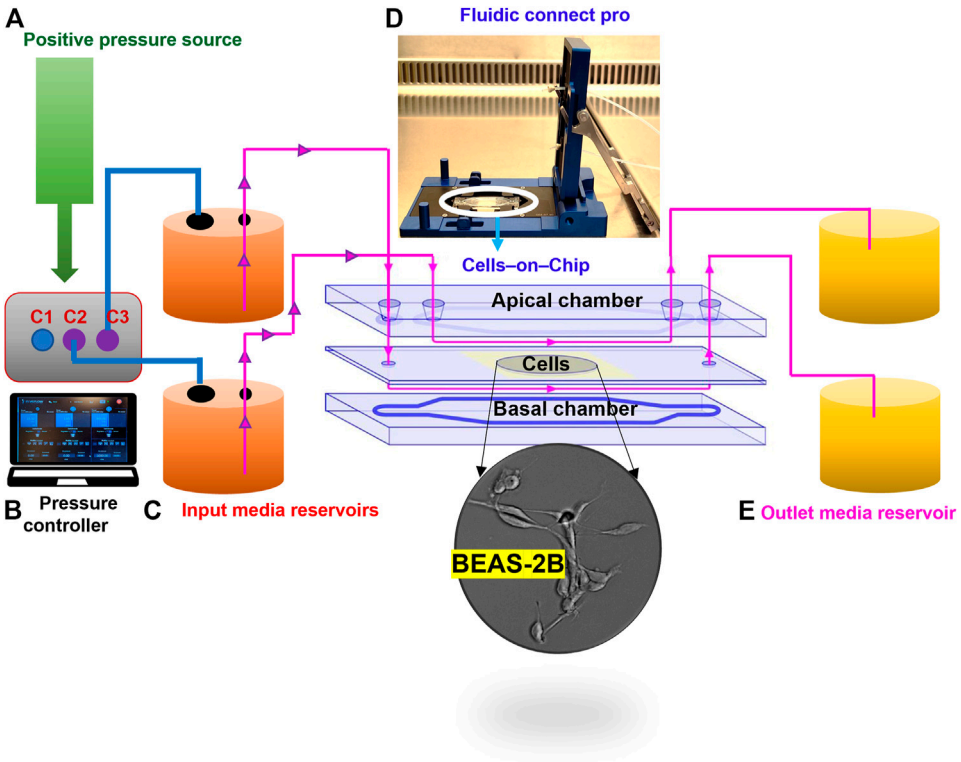

FIGURE 1 | Configuration of the microfluidic cell culture-on-a-chip (COC) platform. The device consists of five different parts: clean air supplied as a positive pressure source **(A)**, for the digitally operated pressure controller with three defined channels to sustain maximum pressures; 10 bar in C1 and 1 bar in C2 and C3 **(B)**; then, the desired amount of pressure supplied to the inlet reservoirs through tubing **(C)**, which enables the flow of medium through the fluidics connect pro device that holds the COC chambers to allow unidirectional flow of medium through the apical and basal sides of the human lung cells in the central cavity **(D)**; finally, flow through is collected in outlet reservoir **(E)**.

were seeded 1 day before the experiment at a density of 60,000 cells/cm^2 either in a 24-well plate or on the microfluidic glass chip for static and dynamic exposures, respectively. The cells grown in 24-well plates and the microfluidic chip were monitored for cell morphology and for cell viability by measuring LDH release, as detailed above. Then, the microfluidic chip was inserted and sealed with top and bottom layers to assemble the COC system. Exposure to SiO_2 and PS NPs (10 μg/ml) was performed under static and dynamic conditions (flow rate: 65 μL/min, shear stress: 0.015 dyne/cm^2). This was achieved by applying a positive pressure (400 ± 7 mbar) in the microfluidic system. The cells were exposed both through the upper and lower channel of the system. For some experiments, exposure was performed separately through upper or lower channel, respectively. After exposure, the samples were collected by trypsinization (0.025%) and fixed with 4% paraformaldehyde for analysis of cellular uptake, see below.

Uptake of Fluorescent Particles by Human Lung Cells

Flow cytometry: The cellular association of SiO_2 NPs or PS NPs with BEAS-2B cells was quantified by measurement of FITC fluorescence by flow cytometry. In brief, the cells were washed thrice and resuspended on HBSS medium and fluorescence intensity was measured using BD LSRFortessa™ flow cytometer (BD Biosciences) operating with BD FACS DIVA™ software (BD Biosciences). The cell population was gated on the basis of side scatter (SSC) and forward scatter (FSC) intensities detected in control samples. To avoid interference from residual NPs or cellular debris, a FSC threshold was set with a cutoff value of 5,000. The data were plotted using FCS Express™ v. 7 Flow Cytometry software and presented in the form of histograms showing a change in fluorescence intensity after NP exposure compared to control. *Confocal microscopy:* To validate the cellular uptake of NPs, samples harvested as described above were analyzed by confocal microscopy. The use of fluorescent NPs and fluorescent dyes precluded the need for antibodies. The formaldehyde fixed cells were washed and stained with phalloidin red (Abcam) for 15 min and counterstained and mounted with ProLong™ Gold Antifade Mountant containing DAPI (Thermo Fisher Scientific) and imaged using a Zeiss LSM880 confocal microscope. Data were also collected along the *z*-axis, and images were further processed using ZEN software (Zeiss).

RESULTS

Cell Culture-on-a-Chip for Assessment of Particle Uptake

We established a microfluidic-based, serum-free bronchial epithelial cells-on-a-chip system for the evaluation of cellular uptake of NPs of differing density. The step wise assembly of the system is described in the **Supplementary Box 1,2**. The configuration of the cell culture-on-a-chip (COC) device is depicted in **Figure 1**. For comparison, cells were maintained in a conventional, 24-well cell culture dish. To allow for the comparison between the two set-ups, we first determined the cell density, cell viability, and cell morphology of the BEAS-2B cells cultured in 24-well cell culture plates vs. in the microfluidic device (**Supplementary Table S1**, **Figure S1**). Notably, cells were seeded at the same cell density in both systems and a low and comparable loss of cell viability (~5%) was noted under both conditions at 24 h. BEAS-2B is a virally transformed yet non-tumorigenic human cell line (Reddel et al., 1988). These cells have been widely used as an *in vitro* model for assaying chemicals and nanomaterials with respect to pulmonary toxicity or carcinogenicity. The BEAS-2B cells were maintained and exposed under completely animal-free conditions in this study meaning that no animal-derived products (such as fetal bovine serum) were applied.

It is well understood that wherever flow occurs (in the body), shear stress exists. Thus, the respiratory epithelium is continuously subjected to shear stresses induced by airflow. It has been estimated that the shear stress values in the nasal cavity during quiet breathing are in the range of 0.5–1.5 dyne/cm^2 (Elad et al., 2006). Higher values may occur as breathing efforts are increased and these may approach the shear stress values that exist in large blood vessels (Elad et al., 2006). Here we applied a constant flow rate (65 μL/min) in the microfluidic system to achieve a shear stress of 0.015 dyne/cm^2. This value is lower than the ones reported for human airways. However, it is noted that the BEAS-2B cells were grown without pre-coating of the substrate with extracellular matrix proteins, as reported previously by others (*e.g.*, Zhao and Klimecki, 2015).

Characterization of Silica and Polystyrene Nanoparticles

Both SiO_2 NPs and PS NPs are widely studied with respect to their biological behavior (Tenzer et al., 2013). Prior to the assessment of cellular uptake, we characterized the NPs in the relevant cell culture medium. Furthermore, we decided to evaluate the NPs before and after passing through the COC (refer to the schematic in **Figure 1**). It is noted that the cell culture medium in the present study is serum-free, yet it remains possible that proteins secreted by the cells, or other cellular metabolites, might influence the NPs (Albanese et al., 2014). Based on the information provided by the suppliers, the SiO_2 NPs and PS NPs displayed similar primary particle sizes (45 and 50 nm, respectively). However, the NPs differed in terms of their density (SiO_2 NPs ~2.0 g/cm^3; PS NPs ~1.06 g/cm^3). Dynamic light scattering (DLS) measurements showed that the hydrodynamic diameter of the SiO_2 NPs in inlet and outlet samples (following 1 h exposure) was 156 ± 1 nm and 123 ± 4 nm, respectively (**Figures 2A,B**). In contrast, the hydrodynamic diameter of PS NPs remained almost identical in the outlet samples (82 ± 1 nm) as compared to the inlet samples (84 ± 1 nm) (**Figures 2D,E**). Furthermore, the ζ-potential was affected both in the case of the SiO_2 NPs and PS NPs when collected from the outlet in comparison to the inlet reservoir, more so for the SiO_2 NPs (**Figures 2C,F**). Hence, the ζ-potential of the SiO_2 NPs was −8.7 ± 2.0 mV and −4.3 ± 0.5 mV in the inlet and outlet samples, respectively (**Figure 2C**) whereas for PS NPs, the ζ-potential was −10.8 ± 1.8 mV and −7.9 ± 0.3 mV in the inlet and outlet samples, respectively (**Figure 2F**).

Dynamic Exposure Conditions Affect Uptake of Particles

It is well established that size, shape, and surface properties of NPs are key determinants for NP interactions with cells and tissues. Furthermore, the density of the particles may also influence the likelihood of cellular interactions, as discussed above. We asked whether human bronchial epithelial cells would take up NPs of varying densities to a different extent under static vs. dynamic exposure conditions. To this end, SiO_2 NPs and PS NPs were used as model NPs. The SiO_2 NPs have a density roughly twice that of blood while the PS NPs are neutrally buoyant (Thompson and Eniola-Adefeso, 2015). BEAS-2B cells were briefly exposed in a conventional, static cell culture model vs. the previously established microfluidic-based COC and uptake was determined using flow cytometry and confocal microscopy. As shown in **Figure 3A**, uptake of the SiO_2 NPs was identical at 2 h under dynamic conditions when compared to static exposure, and this was confirmed by confocal microscopy which revealed ample internalization of clusters of fluorescent NPs under both conditions (**Figures 3B,C**). In the case of the PS NPs (displaying a similar surface charge, but a lower density when compared to the SiO_2 NPs), we observed limited uptake at 2 h under static conditions whereas particle uptake was enhanced under dynamic conditions (**Figure 4A**). This was confirmed by confocal microscopy (**Figures 4B,C**). Cellular internalization of the PS NPs was barely seen under static conditions (at 1 h) (**Figure 4B**). Thus, dynamic exposure to PS NPs enhanced the uptake of the otherwise buoyant NPs.

It is common knowledge that epithelial cells display polarity, characterized by apical and basolateral membrane domains separated by cell junctions. These adherens and tight junctions connect neighboring epithelial cells, while the basal surface interacts with the extracellular matrix through integrin receptors. The apical and basolateral membranes differ in terms of their protein and lipid composition (Cao et al., 2012). We investigated whether apical or basal exposure of NPs under dynamic conditions would influence cellular uptake of NPs. To this end, we exposed BEAS-2B cells in the COC device separately through the upper or lower flow chambers vs. under static conditions. We confirmed that there was less uptake of the PS NPs under static conditions

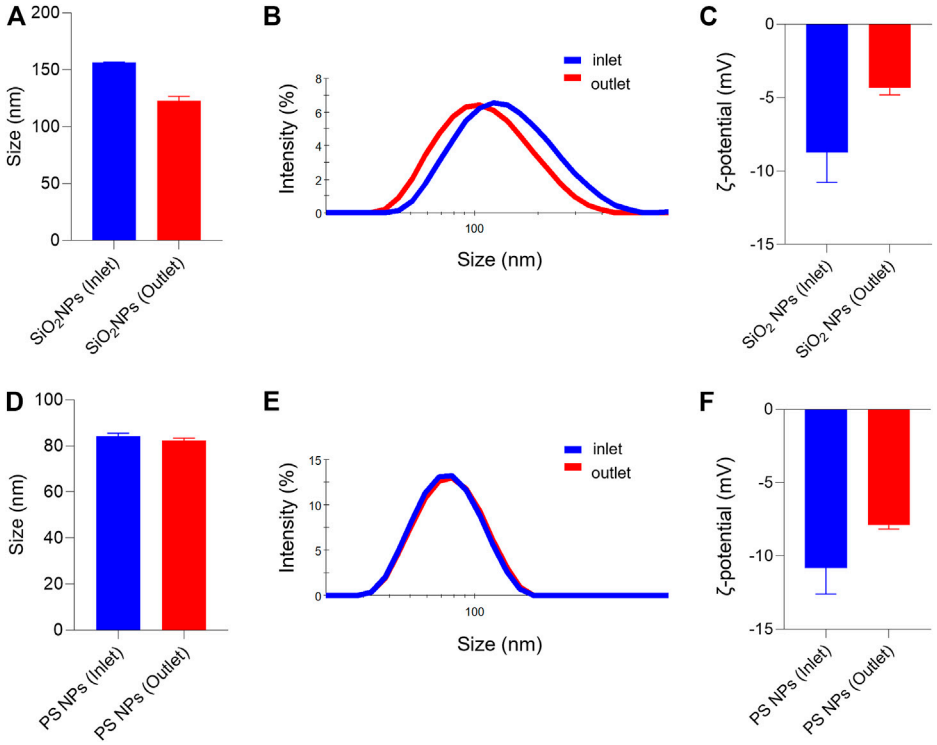

FIGURE 2 | Hydrodynamic diameter and surface charge (ζ-potential) of SiO_2 NPs and PS NPs collected from inlet and outlet reservoirs of the microfluidic device **(A–C)** SiO_2 NPs were evaluated at 2 h of exposure **(D–F)** PS NPs were evaluated after 1 h of exposure. The hydrodynamic diameter of the SiO_2 NPs was: D_h (inlet) = 156 ± 0.5, and D_h (outlet) = 123 ± 4; and for the PS NPs: D_h (inlet) = 84 ± 1.3; and D_h (outlet) = 82 ± 1.0.

(**Figure 5A**). Furthermore, we observed higher uptake of NPs in cells exposed *via* the upper chamber when compared to the lower chamber of the COC device (**Figure 5B**).

DISCUSSION

Most cell culture models are static, and do not reflect the dynamic conditions in a living system. The buoyancy of particles with densities lower or equal to that of cell culture medium poses a problem as they may not reach the cells at the bottom of the dish, leading to an underestimation of their effects on cells (Watson et al., 2016; Stock et al., 2020). Furthermore, several recent studies have shown that the cellular interactions of NPs are dictated by flow rate (Moore, et al., 2017; Yazdimamaghani et al., 2018; Chen et al., 2020). The size and shape of the particles may also come into play (Geng et al., 2007). However, the preferential interaction with spherical vs. elongated particles may be cell type-dependent, as demonstrated in an elegant recent study (Safari et al., 2020). In the present study, we focused on the density of the particles while shape and diameter were comparable. Hence, we tested amorphous SiO_2 NPs (45 nm) and polystyrene (PS) NPs (50 nm), both displaying a negative surface charge in cell culture medium. However, it is important to note that the two tested NPs also may differ in other ways for instance with respect to their surface properties, which could affect the subsequent interactions of NPs with cellular receptors. The detection of nano- and microplastics in biotic and abiotic matrices remains a challenge (Mariano et al., 2021). In the present study, we applied fluorescently labeled NPs for the evaluation of cellular uptake by flow cytometry and confocal microscopy. The two different NPs were internally labeled meaning that the surface properties were not affected by the fluorophore while leaching of the fluorophore is prevented. We used the immortalized human bronchial epithelial cell line BEAS-2B as a model, and the cells were cultivated in serum-free medium; serum, after all, is not a natural biological element of the airways. Overall, our findings suggest that the mode of exposure (i.e., static vs. dynamic) should be considered in order to draw conclusions concerning low-density NPs.

As discussed above, Watson et al. (2016) found that nano-sized polypropylene (PP) particles were cytotoxic only when using an inverted cell culture platform. Similarly, Stock et al. (2020) developed an inverted *in vitro* cell culture system to test micrometer-sized polyethylene (PE) particles and demonstrated that the particles became cytotoxic to HepG2 cells only when exposed in "overhead" cell cultures. Here, we could show higher cellular uptake of PS NPs under dynamic exposure conditions while cellular uptake of SiO_2 NPs was similar under static and dynamic conditions. The present study addressed the cellular uptake of NPs, and the different NPs were tested at a relatively low dose (10 μg/ml) up to 2 h of exposure to avoid overt cell death. However, it is relevant to ask whether enhanced cellular

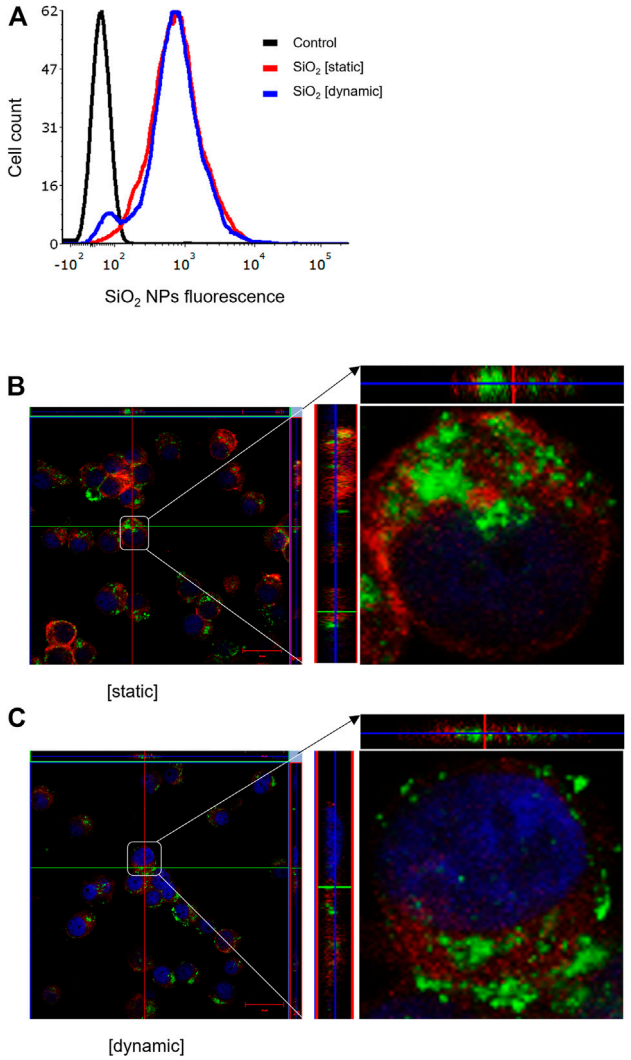

FIGURE 3 | Cellular uptake of fluorescent SiO_2 NPs (10 μg/ml) under static and dynamic exposure conditions. **(A)** Cellular uptake of SiO_2 NPs determined using flow cytometry after 2 h of exposure of BEAS-2B cells **(B,C)** Confocal microscopy of cells exposed for SiO_2 NPs after 2 h under static and dynamic exposures, respectively. The z-stack imaging confirmed the intracellular presence of the NPs. Cells are counterstained using phalloidin red and DAPI (blue) to visualize the actin cytoskeleton and cell nucleus.

uptake of so-called nanoplastics under dynamic exposure conditions also translates into a cytotoxic response. Using a microfluidic device, Oddo et al. (2021) found that the exposure of a human B cell leukemia cell line to polyvinylpyrrolidone (PVP)-coated silver NPs under dynamic conditions resulted in a 3-fold increase in toxicity compared to static conditions. Further studies are needed to address whether this also holds true for so-called nanoplastics.

We have previously shown, using primary human monocyte-derived macrophages as a model, that surface coating (i.e., the intrinsic "identity") as well as protein adsorption (the acquired "identity") both affect the cellular uptake of magnetic NPs (Vogt et al., 2015). Furthermore, other investigators have shown that protein corona formation on the surface of lipid NPs is influenced by dynamic flow conditions, which may, in turn, affect uptake of the NPs in various cancer cell lines (Palchetti et al., 2017). In a more recent study, Srivastava et al. (2020) established a microfluidic-based system for the real-time monitoring of protein corona formation using carbon NPs displaying different surface properties. However, we maintained the cells in FBS- and BPE-free cell culture medium. This does not preclude the formation of a bio-corona derived from the cellular secretome, although this remains purely hypothetical at present. In fact, our DLS measurements at the inlet vs. the outlet of the microfluidic device demonstrated that the hydrodynamic diameter of the PS NPs was identical before

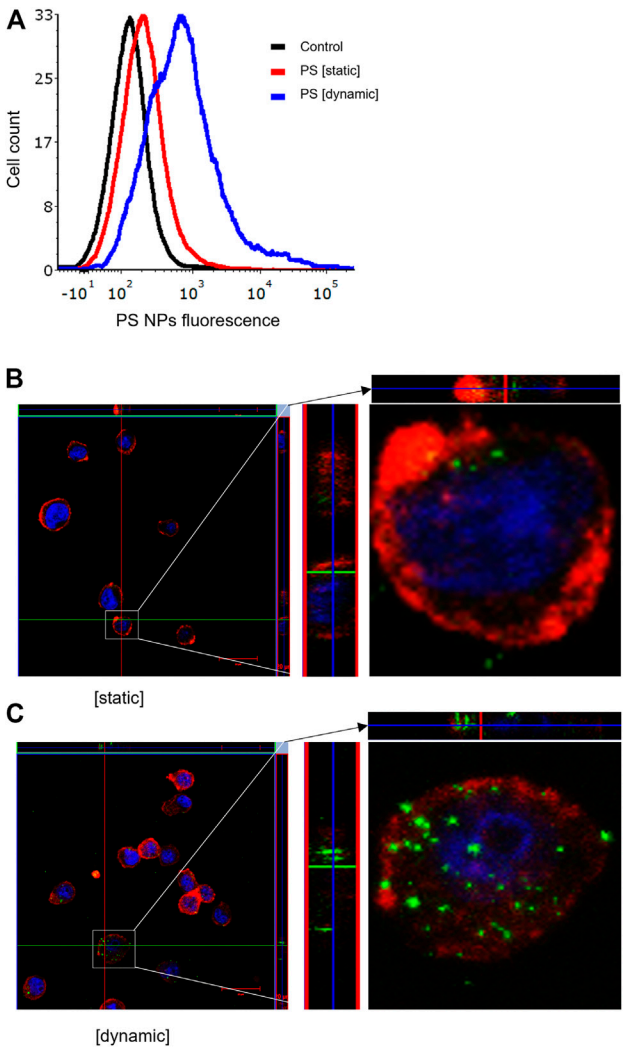

FIGURE 4 | Cellular uptake of fluorescent PS NPs (10 μg/ml) under static and dynamic exposure conditions. **(A)** Cellular uptake of PS NPs determined using flow cytometry after 2 h of exposure of BEAS-2B cells **(B,C)** Confocal microscopy of cells exposed for PS NPs after 1 h under static and dynamic exposures, respectively. The z-stack imaging confirmed the intracellular presence of the NPs. Cells are counterstained using phalloidin red and DAPI (blue) to visualize the actin cytoskeleton and cell nucleus.

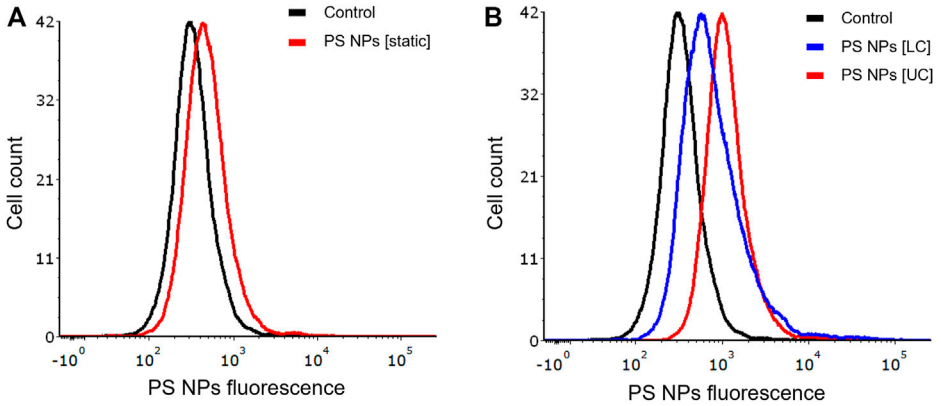

FIGURE 5 | Role of cell polarity. BEAS-2B cells showed differential uptake of PS NPs (10 μg/ml) upon exposure through the upper and lower channels of the microfluidic device. **(A)** static conditions (conventional 24-well plate); **(B)** dynamic exposure using the COC platform. Cells were exposed for 1 h and the samples were analyzed by flow cytometry.

and after exposure. Therefore, we may conclude that the differences in uptake of PS NPs under static and dynamic conditions is related to the flow and not to corona formation (Tenzer et al., 2013). It is pertinent to note that shear stress may also affect the cells themselves, and not only the way in which particles interact with the cells. Hence, Kim et al. (2011) investigated silica NPs using a microfluidics system and found that the NPs showed higher toxicity towards endothelial cells under flow conditions. The authors argued that these differences resulted from the shear stress rather than dose, one potential explanation being that increased shear stress triggers the activation of endothelial cells.

Bronchial epithelial cells serve as a barrier to pathogens and these cells are endowed with innate immune receptors including the so-called Toll-like receptors (TLRs) (Gliga et al., 2020). It is currently not known how nanoplastics gain access to cells, and whether specific cell surface receptor(s) are involved. However, we provided evidence suggestive of selective uptake of PS NPs *via* the apical cell membrane of BEAS-2B cells as opposed to the basolateral surface. This is not surprising as the apical membrane is normally confronted with the external environment (Cao et al., 2012). Further studies are needed to explore differences in endocytosis or phagocytosis of NPs at the apical and basolateral membranes in bronchial epithelial cells. It also remains to be proven whether BEAS-2B cells are truly polarized (Papazian et al., 2016). Previous studies have shown that uptake of simian virus 40 (SV40) by polarized epithelial cells is restricted to the apical membrane implying that its receptors are non-uniformly expressed in polarized cells (Clayson and Compans, 1988; Basak et al., 1992). Furthermore, several TLRs are expressed in epithelial cells of the intestinal tract. Interestingly, Lee et al. (2006) found that activation of TLR9 through the apical and basolateral surface of intestinal epithelial cells leads to distinct responses. This illustrates the critical importance of polarity of epithelial cells and shows that receptors may be expressed differently at the apical and basolateral membranes or, alternatively, that the same receptor may trigger distinct responses depending on the polarity of the cells.

CONCLUSIONS

It has been shown that plastic particles float in cell culture medium and thus do not reach the cells under standard *in vitro* exposure conditions. We established a microfluidic-based platform that allows for dynamic exposure of cells. The human lung cells were maintained under completely animal-free conditions (*i.e.*, no animal-derived products such as fetal bovine serum or antibodies were used). Using this model, we studied the uptake of SiO_2 NPs and PS NPs in cells under dynamic and static exposure. We observed higher uptake of PS NPs under dynamic conditions. These findings suggest that exposure conditions need to be adjusted to mimic the physiological conditions of shear stress especially when dealing with low-density particles. This is relevant not only for the safety assessment of nano- and microplastics, but also in nanomedicine, as shear stress may also dictate the interaction of drug-loaded NPs with cancer cells (Tee et al., 2019). Thus, standard *in vitro* methods based on static cell culture may not be suitable for studies of low-density (buoyant) particles, and may, in fact, underestimate the cellular uptake/impact of such particles, as shown here.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

BF and KB conceived the project and secured the funding; RB prepared fluorescent particles, GG and SV performed

experiments and analyzed data, and GG drafted the paper; BF analyzed data and edited the article; all co-authors approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2021.735331/full#supplementary-material.

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Primary and Secondary Genotoxicity of Nanoparticles: Establishing a Co-Culture Protocol for Assessing Micronucleus Using Flow Cytometry

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Genotoxicity is an important endpoint to assess for understanding the risks associated with nanoparticles (NPs). Most genotoxicity studies performed on NPs have focused on primary genotoxicity analyzed by comet- or micronuclei (MN) assay using microscopic scoring. Here, we established a protocol for a more efficient version of MN assessment using flow cytometry and, importantly, both primary and secondary (inflammation-driven) genotoxicity was assessed. Human bronchial epithelial cells (HBEC-3kt) were exposed to nickel oxide (NiO) NPs directly or indirectly. The indirect exposure was done to assess secondary genotoxicity, and in this case immune cells (THP-1 derived macrophages) were exposed on inserts and the HBEC were cultured in the lower compartment. The results in monocultures showed that no increased MN formation was observed in the HBEC cells but instead a clear MN induction was noted in THP-1 cells indicating higher sensitivity. No MN formation was either observed when the HBEC were indirectly exposed, but an increase in DNA strand breaks was detected using the comet assay. Taken together, the present study emphasizes the feasibility of assessing primary and secondary genotoxicity and, furthermore, shows a clear MN induction in THP-1 monoculture following NiO NPs exposure.

Keywords: NiO nanoparticles, HBEC3-kt cells, THP-1 cells, macrophages, cell cycle analysis, DNA damage, micronuclei, metal oxide

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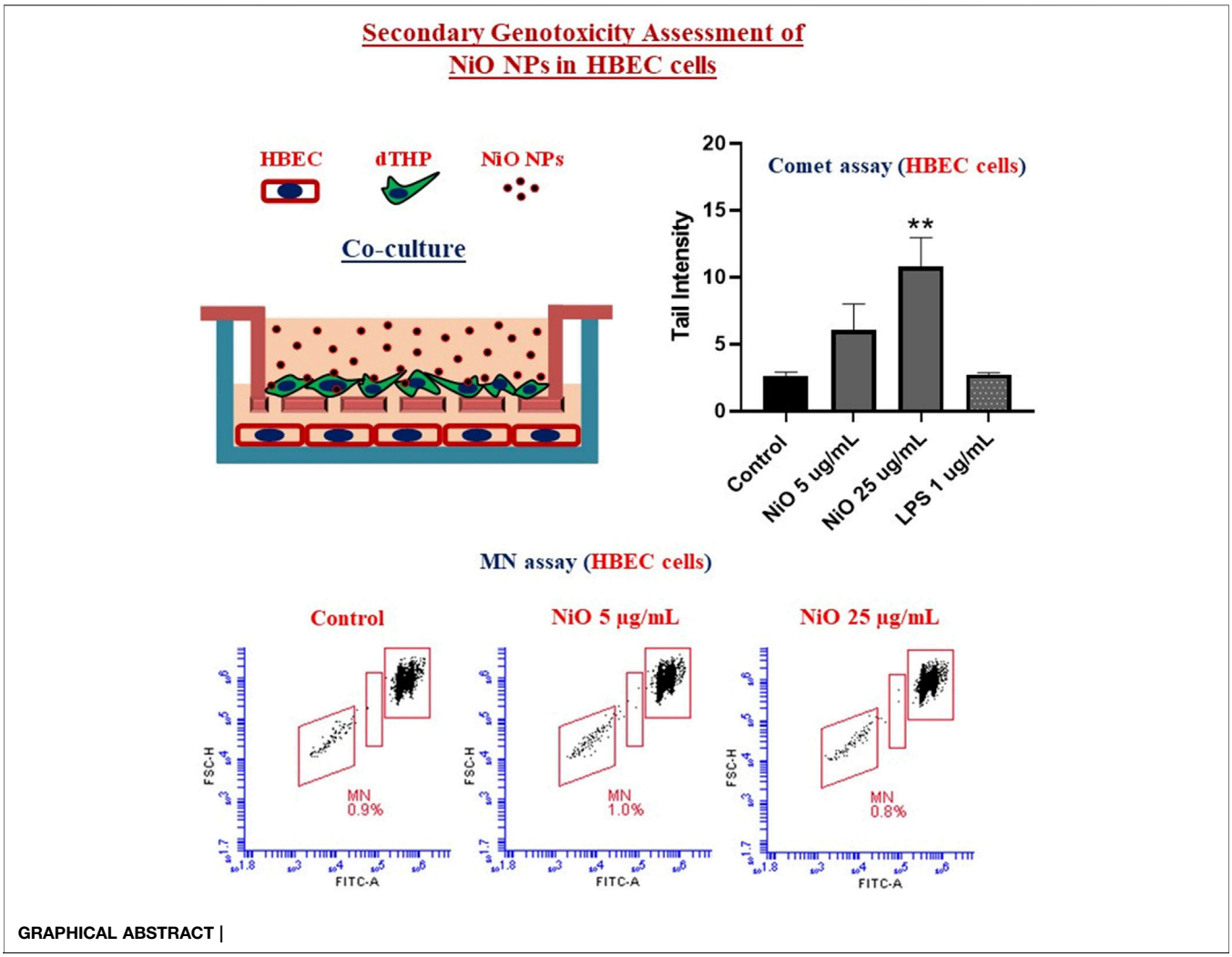
INTRODUCTION

Nanoparticles (NPs) possess distinct physicochemical properties, and their unique characteristics makes them novel entities for a wide range of applications in medicine, engineering, pharmaceuticals, cosmetics, and electronics etc., (Salata, 2004; Ealia and Saravanakumar, 2017). However, their extensive production and usage lead to a demand for toxicity evaluation as well as assessment of health risks at occupational and environmental settings (Kessler, 2011; Batley et al., 2013). Among the various NPs, nickel oxide (NiO) is vastly employed in industrial applications such as metal printing, electronics, ceramics, catalysis, and sensing (Zhou et al., 2017; Sousa et al., 2018; Dumala et al., 2019; Jaji et al., 2020; Taeño et al., 2021; Wang et al., 2021). Moreover, exposure is common at nickel refineries, metal alloy production sites and at occupational setting where welding is performed (Klein and Costa, 2015; Pesch et al., 2019). The health risks possibly caused by inhalation of nickel is evident since nickel compounds are classified as carcinogenic to humans (Group 1) by the International Agency for

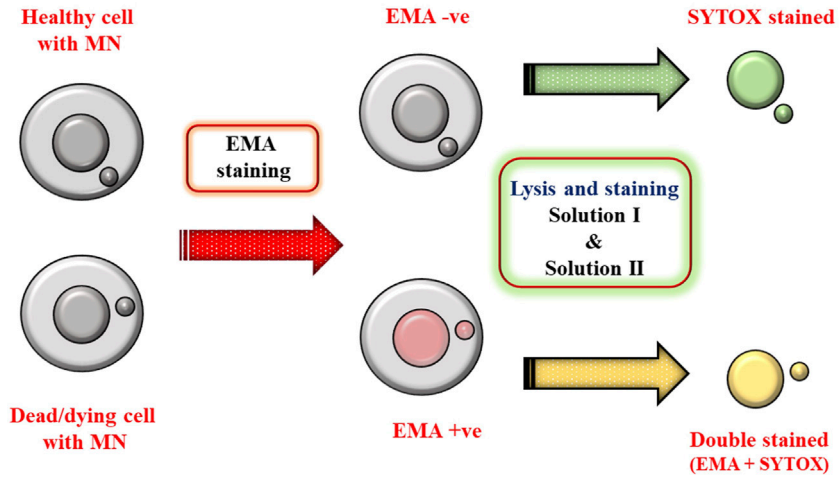


GRAPHICAL ABSTRACT |

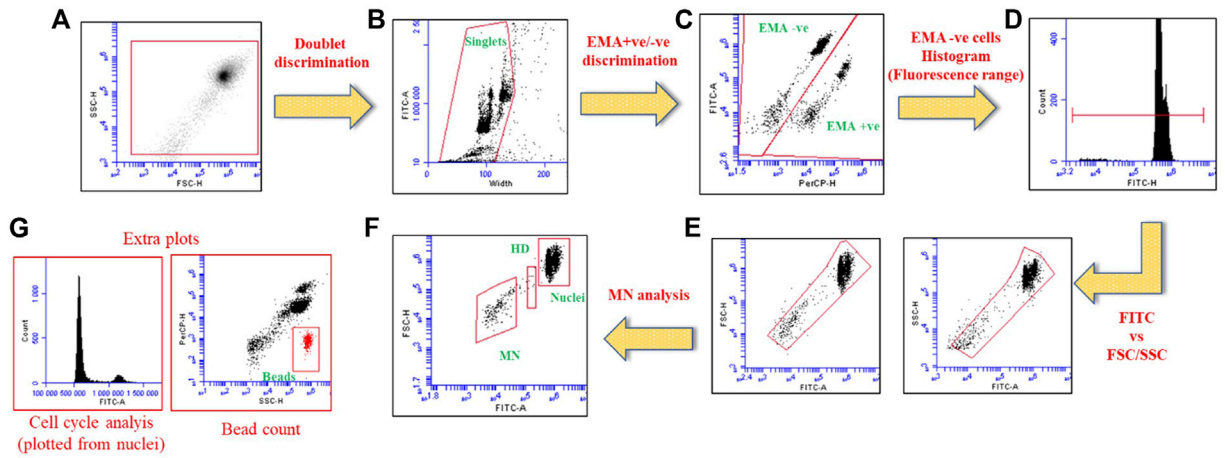
Research on Cancer. Furthermore, several studies have shown genotoxicity following exposure of lung cells to NiO NPs (Capasso et al., 2014; Di Bucchianico et al., 2018; Akerlund et al., 2019).

Genotoxicity testing typically includes a battery of assays and in a first tier testing various *in vitro* assays are performed (Dusinska et al., 2019). The most commonly used assays in genotoxicity testing of NPs are Micronucleus (MN) assay and Comet assay, respectively (Magdolenova et al., 2014). They are often used in combination due to their advantages over each other, where comet assay detects the DNA damage/stand breaks with high sensitivity, and MN assay can differentiate the aneugenic and clastogenic effects (Magdolenova et al., 2014; Franz et al., 2020). In the conventional MN method microscopic scoring is mainly used to score at least 2000 binucleate cells per concentration. This evaluation is tedious and a time-consuming process. Hence, high throughput methods like flow cytometric MN analysis can be employed to automate the analysis. Furthermore, the sequential staining procedure allows discriminating the actual MN compared to MN originated from dead or dying cells (necrotic/apoptotic population) (Avlasevich et al., 2006; Bryce et al., 2007).

One aspect often not considered in standard genotoxicity assessment is the so-called secondary genotoxicity. In general, secondary genotoxicity is exhibited *in vivo* via inflammation mediated mechanisms caused by activation/recruitment of phagocytes (macrophages or neutrophils). For instance, the presence of foreign bodies or uncleared NPs in lung cells can elicit a chronic immune response involving oxidative stress (ROS and reactive nitrogen species). The whole cascade triggers free radical generation, and cytokine/chemokine release from immune cells causing secondary genotoxicity towards neighboring target cells. Some advanced *in vitro* approaches have been used to mimic the *in vivo* conditions and understand the secondary genotoxicity mechanisms (Evans et al., 2017; Akerlund et al., 2019; Evans et al., 2019; Burgum et al., 2021). These approaches include application of conditioned culture medium from one cell type to other target cells, co-culture systems to facilitate cell-to-cell interplay among different cell types, and complex 3D cellular microtissues (spheroids or



SCHEME 1 | Schematic describing the advantage of differential staining in excluding dead/dying cells (EMA +ve population).



SCHEME 2 | Dot plots and histograms illustrating the analysis setup for MN detection using flow cytometric method. **(A)** Total gated population, **(B)** Double discrimination to choose singlet nuclei, **(C)** EMA +ve/–ve discrimination to choose EMA-ve nuclei **(D)** Histogram representing EMA –ve population with marker to cover nuclei and MN population, **(E)** Fluorescence vs. FSC/SSC dot plots to gate the free nuclei, **(F)** The final dot plot generated from the plots "E" to determine the nuclei, MN and hypodiploid populations (HD) respectively. **(G)** Additional plots to analyze cell cycle disruptions, and bead count can be used to determine the relative survival rate (using nuclei to bead ratio).

organoids) models which resemble *in vivo* tissue architecture and characteristics. However, only a limited number of studies have attempted to investigate secondary genotoxicity (Evans et al., 2017; Akerlund et al., 2019; Evans et al., 2019). Similarly, only few studies have used flow cytometry for more efficient analysis of MN induction following exposure to nanoparticles (Di Bucchianico et al., 2017; Lebedova et al., 2018). The aim of this study was to establish a flow cytometry protocol for MN analysis that is useful for detecting primary and secondary genotoxicity of NPs. A co-culture model with macrophages and lung cells was used to determine the secondary genotoxicity of NiO NPs. The MN formation using the flow cytometer approach was also compared to the results achieved with the comet assay for assessment of DNA strand breaks. Importantly, possible interferences were also considered.

MATERIALS AND METHODS

Cell Culture

HBEC3-kt (Human bronchial epithelial cells) were originally obtained from American Type Culture Collection (ATCC) and were cultured in 50% LHC-9 (Laboratory of Human Carcinogenesis-9, Gibco, Carlsbad, CA) and 50% RPMI medium (Roswell Park Memorial Institute, Sigma Aldrich, St. Louis, MO) without serum and supplemented with 1% penicillin-

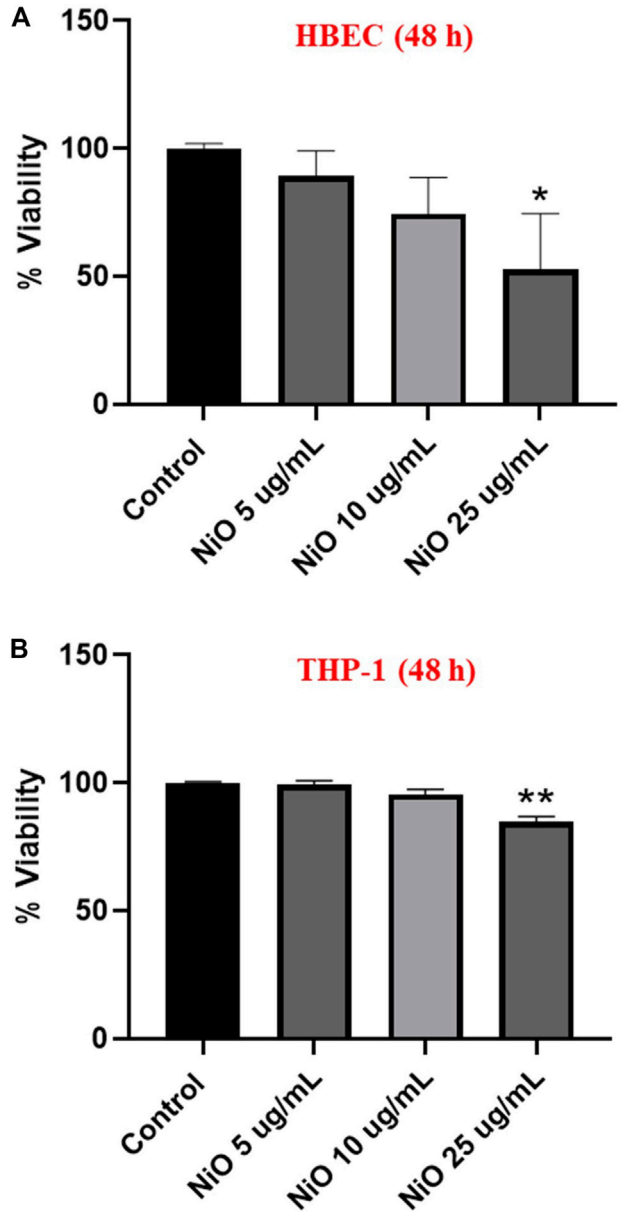


FIGURE 1 | Cytotoxicity assessment of NiO NPs following exposure of HBEC cells **(A)** and THP-1 cells **(B)** after 48 h. The bars represent mean ± SEM. Asterisks indicate significance (*$p < 0.05$) compared to untreated cells (control).

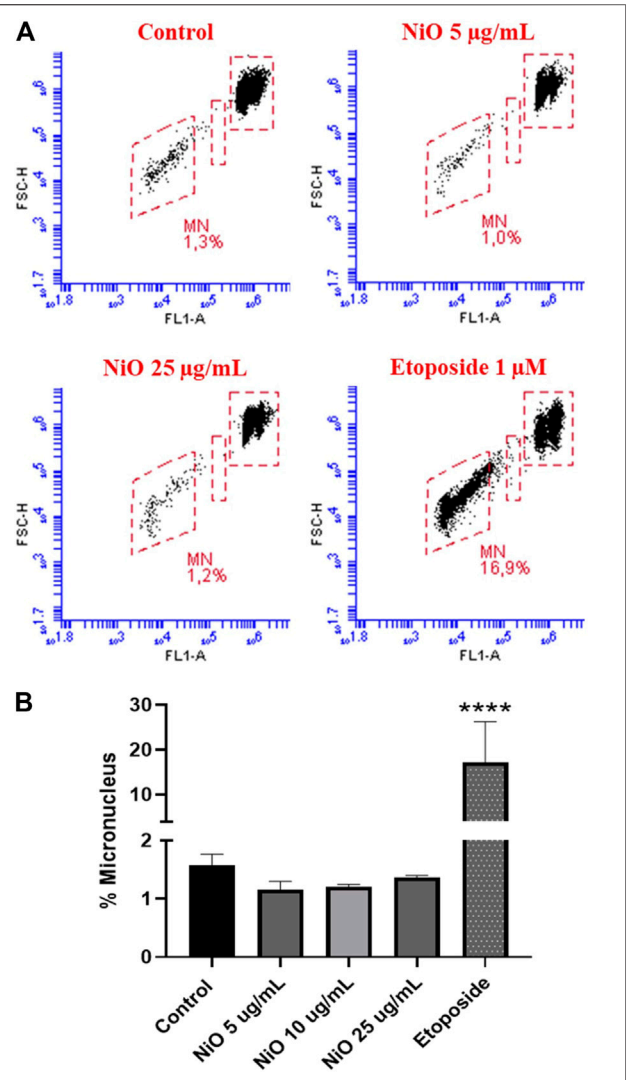


FIGURE 2 | Micronucleus detection in HBEC cells exposed to NiO NPs after 48 h exposure using flow cytometric method. **(A)** Dot plot representing MN formation and **(B)** bar plot indicating MN% expressed from three individual experiments. Etoposide (1 µM) was used as a positive control. The bars represent mean ± SEM. Asterisks indicate significance (*$p < 0.05$) compared to untreated cells (control).

streptomycin (Gibco, Buffalo, NY) and 2 mM L-glutamine (Gibco, Buffalo, NY). Prior to cell culture T75 flasks were coated with 3 mL of collagen (0.032 mg/mL, Type I, PureCol®, Advanced BioMatrix Carlsbad, CA) for 2 h, and cells were maintained at 37°C in a humidified incubator supplied with 5% CO_2.

THP-1 monocytes (THP) were obtained from Sigma-Aldrich and cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin. Cells were grown in a T75 cm^2 flask (VWR 734-2313) and incubated at 37°C in a humidified incubator supplied with 5% CO_2. Cell density was maintained between 5×10^5–1.5×10^6 cells/mL. THP were differentiated to macrophages (dTHP) with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) at 37°C for 48 h.

Particle Preparation and Characterization

NiO NPs (<50 nm diameter, >99.8% purity, Cat# 637130, 17198PJ) were purchased from Sigma-Aldrich (St. Louis, MO). NPs were weighed and dispersed in Milli-Q water to make a stock concentration of 1 mg/mL. The suspension was then sonicated in a water bath sonicator (VWR, USC 200T) for 20 min at 30°C, and then further diluted in cell medium to the indicated concentrations. Detailed characterization of the NiO NPs has

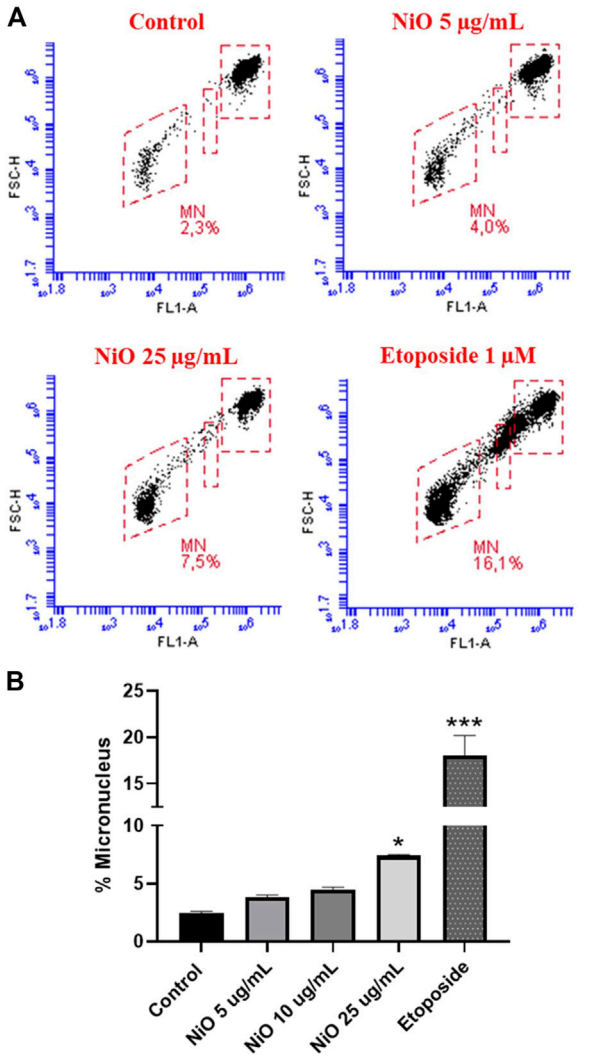


FIGURE 3 | Micronucleus detection in THP-1 cells exposed to NiO NPs for 48 h using a flow cytometric method. **(A)** Dot plot representing MN formation and **(B)** bar plot indicating MN% expressed from three experiments. Etoposide (1 µM) was used as positive control. The bars represent mean ± SEM. Asterisks indicate significance (*$p < 0.05$) compared to untreated cells (control).

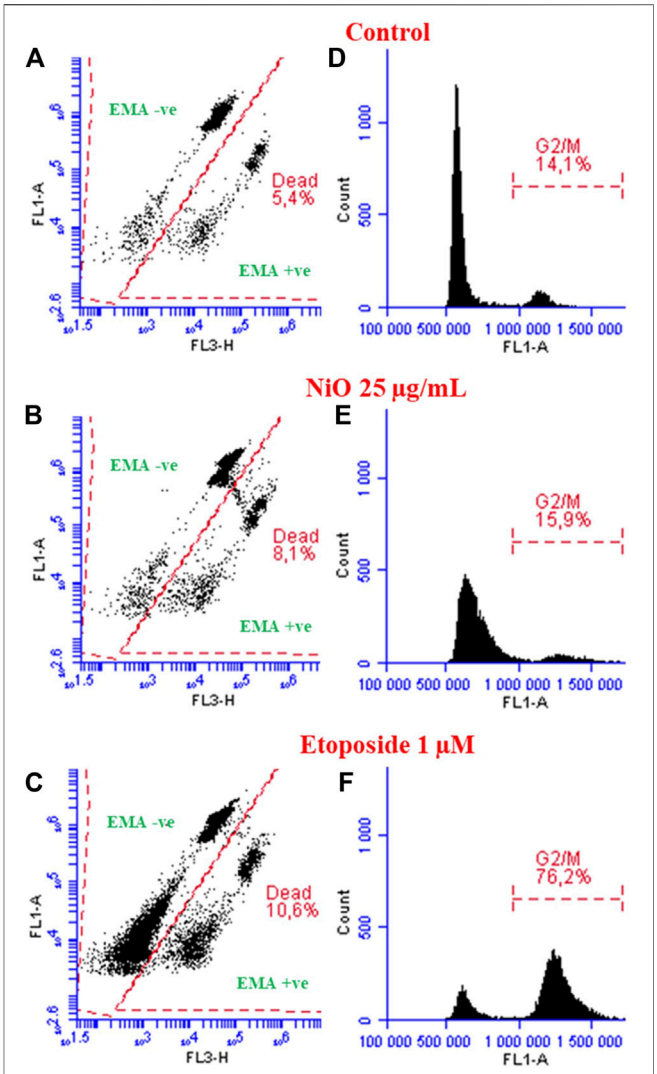


FIGURE 4 | Analyzing dead population and cell cycle perturbations (HBEC cells) after exposure to NiO NPs and etoposide after 48 h. Dot plots displaying dead nuclei population (EMA +ve cells) after respective treatments compared to control **(A–C)**. Histograms represent cell cycle analysis, particularly G2/M arrest shown after NiO NPs and etoposide compared to control **(D–F)**.

been presented in our earlier publications (Di Bucchianico et al., 2018; Akerlund et al., 2019).

Alamar Blue Assay

THP-1 cells were seeded at a density of 2.0×10^4 cells/well in a 96 well plate. HBEC cells (1.0×10^4/well) were seeded in a collagen precoated 96 well plate and incubated for 24 h. Both cell types were exposed to NiO NPs at 5, 10, and 25 µg/mL for 48 h in their respective medium and cell culture medium was used as a negative control. After exposure, supernatant was removed from HBEC and 10% Alamar Blue (Invitrogen, Carlsbad, CA) prepared in fresh medium was added. In case of THP-1 cells, Alamar blue was added directly into the existing medium (to make a final concentration of 10%) and incubated for 2 h at 37°C. Wells containing only 10% Alamar blue and NiO NPs were included to rule out particle interference in the study. After incubation, the fluorescence was read at 560 nm excitation and 590 nm emission using a microplate reader (Tecan, San Jose CA, Infinite F 200, Software: Magellan 7.2). Negative control was normalized to 100% viability and treated samples were compared with this value.

Co-Culture Using Inserts

The differentiation of THP-1 to macrophages (dTHP) was carried by incubating 5.0×10^5 cells/insert in 400 µL of medium containing PMA. Inserts [ThinCert™ PET membrane inserts (Greiner Bio-One, 662641), pore size 0.4 µm, surface 0.33 cm^2]

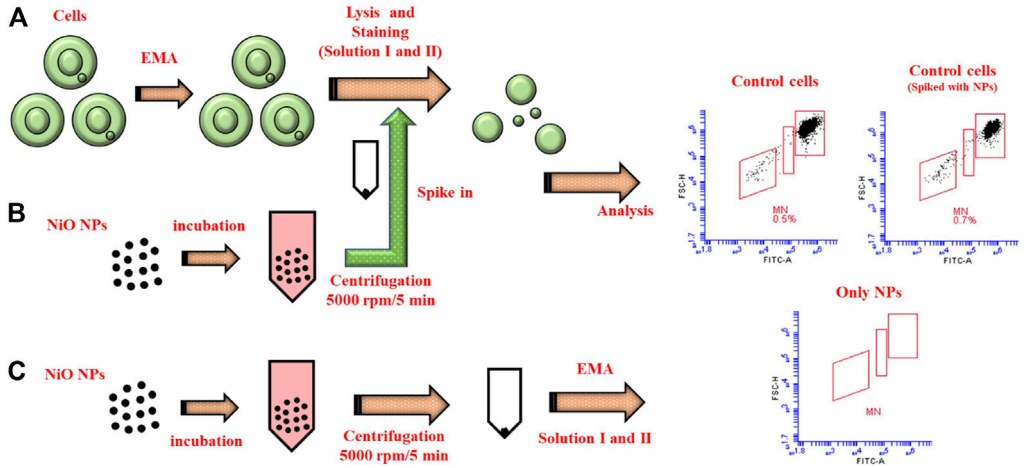


FIGURE 5 | Schematic illustrating the reliability of MN protocol. **(A)** MN staining procedure carried in control cells after 48 h incubation at 37°C. **(B)** NiO NPs were incubated separately, collected through centrifugation, and spiked with the control cells to compare the interferences in MN count. **(C)** Only NiO NPs were stained following the same MN protocol and analyzed to understand their spread in the MN dot plots.

with cells were placed in a 24 well plate and allowed to differentiate for 48 h. HBEC cells at a density of 0.6×10^5 cells (600 µL/well) were seeded in 24 well plate and left for 24 h, fresh HBEC medium was replaced before placing the inserts. Prior to exposure, dTHP were washed gently with PBS and inserts were placed on top of HBEC cells. The dTHP were then exposed to NiO NPs for 24 or 48 h in RPMI medium, unexposed cells were considered as negative control.

After exposure, inserts were removed and dTHP cells were preceded for cytotoxicity testing. Alamar blue was added to the medium, after 2 h supernatant from inserts was transferred to a 96 well plate and fluorescence intensity was recorded. HBEC cells were assessed for secondary genotoxicity using micronucleus and comet assays.

Micronucleus Assay

Micronucleus detection was followed by a flow cytometric method described by Bryce et al., with some modifications (Bryce et al., 2007).

Monocultures and Primary Genotoxicity

HBEC cells (0.6×10^5 cells) were exposed to NiO NPs (5, 10, and 25 µg/mL) for 48 h in a 24 well plate. After treatment cells were washed with chilled PBS and continued to step 1–3 as described below. In case of suspension cultures (THP-1) 1.2×10^5 cells were seeded in a 24 well plate, and after NPs incubation cells were centrifuged at 1,500 rpm for 5 min. Supernatant was discarded, cells were washed with PBS and centrifuged again to collect the pellet. Further, cells were processed to step 1–3 before analysis.

Coculture and Secondary Genotoxicity

After exposure to NiO NPs (5 and 25 µg/mL) the inserts containing dTHP were removed and, HBEC cells from co-culture were transferred on to ice and left for 20 min. Next the medium was removed, and cells were washed with ice-cold PBS.

Step 1: Ethidium Monoazide Bromide dye (EMA, Invitrogen) stock was prepared in DMSO, and the working concentration (10 µg/mL) was prepared in buffer solution (PBS+2% FBS). EMA solution (300 µL) was added to cells and incubated on ice for 30 min, under a cool white light. After incubation cells were washed with ice-cold buffer solution and continued for step 2.

Note: For suspension culture (THP-1), after EMA staining cells were centrifuged at 1,500 rpm for 5 min and pellet was dispersed in buffer solution to wash. Thereafter, cells were centrifuged to collect the cell pellet and processed for step 2.

Step 2: Lysis solution I (Trisodium citrate 1.0 mg/mL; NaCl 0.584 mg/mL; Igepal 0.6 µL/mL; RNase A 100 µg/mL and SYTOX Green 0.5 µM) was prepared in Milli Q and filtered using 0.22 µm pore size membrane filter. To each well 300 µL of lysis buffer (solution I) was added and incubated in dark for 1 h at room temperature.

Step 3: Solution II (Citric acid 15 mg/mL; sucrose 85.6 mg/mL and SYTOX Green 0.5 µM) was prepared in Milli Q and filtered using 0.22 µm pore size membrane filter. 300 µL of solution II was added to the cells (without discarding solution I) and was allowed to equilibrate in dark for 30 min at room temperature.

Additional step: A drop of cell sorting set-up beads (6 µm, for blue lasers, Invitrogen) can be mixed in solution II (~10 mL) prior adding to cells. Based on the healthy nuclei to bead ratio, cytotoxicity can be calculated from the relative survival values.

Advantage of Differential Staining

EMA dye enters the cells which have compromised membrane and binds covalently to nucleic acids after photolysis. Up on binding to nucleic acids, its fluorescent intensity increases and

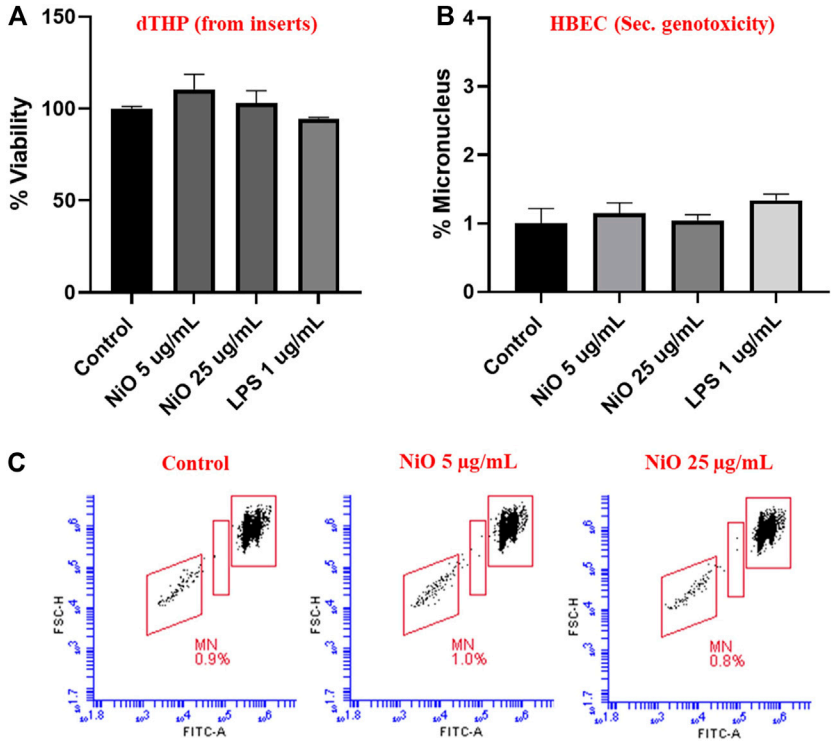


FIGURE 6 | Secondary genotoxicity evaluation in HBEC cells using Flow MN method. Inserts containing dTHP were separated from co-culture and Alamar blue assay was carried to determine the cell viability after NiO NPs exposure for 48 h **(A)**. MN analysis performed in HBEC cells from the co-culture model to evaluate the secondary genotoxicity after 48 h, **(B)** bar plot representation of MN% from three experiments, and **(C)** Dot plot presentation of MN analysis from a single experiment shown in the bar plot. The bars represent mean ± SEM.

can differentiate live and dead cells in a mixed population. After EMA staining, the detergent in solution lyses the cytoplasmic membrane of the cells and liberates nuclei and MN. Concomitantly, SYTOX Green stains the overall DNA and this differential staining procedure helps to rule out the dead/dying cells (double positive) compared to healthy cells. Based on staining, healthy cells are termed as EMA-negative (EMA –ve) and dead cells as EMA-positive (EMA +ve) population. For Flow cytometric analysis, only EMA –ve nuclei were considered for MN evaluation to exclude necrotic or apoptotic population (see **Scheme 1**).

After incubation cells were acquired using BD Accuri™ C6 (BD Biosciences) at 488 nm excitation. EMA-associated, and SYTOX Green fluorescence were recorded in FL3 (610/20 nm) and FL1 channel (530/30 nm). In total, 10,000 gated nuclei were acquired per sample and data analysis was performed with BD Accuri™ C6 Software. Representative plots considered for MN analysis are presented in **Scheme 2**.

Evaluating Cell Cycle Perturbations

In addition to nuclei and MN detection, SYTOX Green fluorescence can be utilized to determine the cell cycle information. The gated "nuclei" population is used to analyze the cell cycle effects (see **Scheme 2**).

NPs Spiking to Determine the Interferences With MN Analysis

In order to test for possible NPs interference with MN analysis, additional experiments were performed on nuclei from control cells. Thus, unexposed THP-1 cells (seeded at 1.2×10^5/well) incubated at 37°C for 48 h, were centrifuged at 1,500 rpm for 5 min. Supernatant was discarded, cells were washed with chilled PBS and centrifuged to collect the pellet. Next, cells were stained with EMA followed by lysis in solution I. Along with Solution II, NiO NPs (incubated in medium at 37°C for 48 h, centrifuged at 5,000 rpm for 5 min to collect the pellet) were added and flow cytometer analysis was then performed. In addition, NiO NPs (without any nuclei) was processed for EMA staining, solution I and Solution II, similar to the protocol mentioned. These NPs were analyzed directly to determine the particle location, fluorescence in the plots, and to compare their range with MN and nuclei populations.

Comet Assay

HBEC cells from the co-culture were assessed for DNA strand breaks by alkaline single cell comet assay as previously described (Gliga et al., 2014). Briefly, cells were mixed with 0.75% agarose (Sigma-Aldrich, St. Louis, MO) maintained at 37°C and pipetted onto precoated (0.3% agarose) microscopic slides. After gelling,

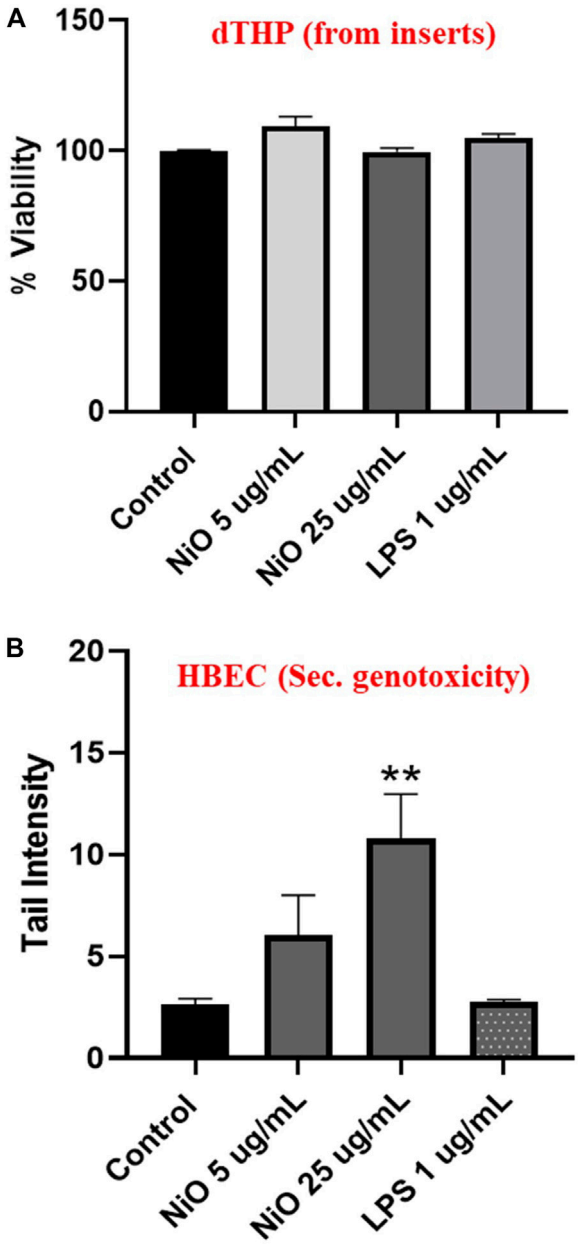


FIGURE 7 | Secondary genotoxicity evaluation in HBEC cells using comet assay. Inserts containing dTHP were separated from co-culture and alamar blue assay was carried to determine the cell viability after NiO NPs exposure for 24 h **(A)**. Bar plot representing DNA damage in HBEC cells from the co-culture model to evaluate the secondary genotoxicity after 24 h **(B)**. The bars represent mean ± SEM. Asterisks indicate significance (*p < 0.05) compared to untreated cells (control).

the slides were transferred into lysis buffer containing 1% Triton X-100 and left overnight at 4°C. Next, slides were placed in electrophoresis buffer to unwind the DNA for 20 min, and electrophoresis was performed at 29 V for 30 min. The slides were moved into neutralizing buffer, washed in Milli-Q water, and dried overnight. Thereafter, cells were fixed in methanol for 5 min and stained with diluted (1:10,000) SYBR Green (Life Technologies™, Carlsbad, CA) in Tris-acetate-EDTA (TAE) buffer for 20 min. Slides were washed once with TAE buffer and allowed to dry before imaging. Slides were scored using a fluorescence microscope (Leica DMLB, Wetzlar, Germany) equipped with Comet Assay IV software. In total, 50 comets were counted for each sample and the DNA damage was represented as % of DNA in tail. Comets appearing as "hedgehogs" were few and were not scored. These are sometimes viewed as dead/dying cells but can also indicate damage that can be repaired (Lorenzo et al., 2013), and such comets may not be recognized by image analysis.

Metal Release From Inserts Analyzed by ICP-MS

The metal/ion release from the inserts into lower compartment was analyzed using an inductively coupled plasma mass spectrometer, ICP-MS (ICAP Q; Thermo scientific, Waltham, MA, United States). In brief, dTHP cells (5×10^5/insert) were exposed to NiO NPs at 25 μg/mL and were placed over HBEC cells for 48 h at 37°C. After incubation inserts were removed, and medium (containing dissolved Ni or possibly NiO that may be transported from the upper compartment) was collected from the HBEC wells and stored at 4°C prior to analysis. Furthermore, to analyze transport of Ni over the insert without cells, NiO NPs (25 μg/mL) were added to inserts without dTHP (acellular control) and medium was collected from HBEC cells after incubation. For analysis, samples were diluted 10 times in 2% HNO_3 and in similar standard solutions of Ni were prepared (0, 0.1, 1, 5, 10, 50, 100, 500 ppb in 2% HNO_3). Indium was added as an internal standard to all samples equally (5 μg/L), to enable the measured metal concentrations based on its recovery. The levels of ^{58}Ni and ^{115}In were quantified in each sample acquired in KED mode using argon as vector gas and helium as collision gas. The recovery of internal standard was observed between 80 and 100%. The limit of detection (LOD) was evaluated as 3 x standard deviation of blank medium samples.

RESULTS

Cytotoxicity

Cell viability was assessed by using Alamar blue assay in HBEC and THP-1 monocultures after 48 h exposure to NiO NPs. In HBEC and THP-1 cells, a significant cytotoxic effect was observed at the dose 25 μg/mL NiO (**Figures 1A,B**).

Primary and Secondary Genotoxicity Assessed as MN Induction Using Flow Cytometer

Primary genotoxicity: MN formation was evaluated after 48 h exposure of HBEC and THP-1 cells to different concentrations of NiO NPs (5, 10, and 25 μg/mL). The results showed no significant change in MN induction in HBEC cells (**Figures 2A,B**), whereas in THP-1 cells, a clear increase was observed (7.5% MN) at 25 μg/mL compared to control (2.3% MN) (**Figure 3**). The positive

control etoposide (1 μM) caused a significant increase in MN formation in both HBEC (16.9% MN) and THP-1 cells (16.1% MN) after 48 h exposure (**Figures 2**, **3**).

The MN protocol along with micronucleus detection has an advantage to evaluate the cytotoxicity and cell cycle changes. EMA-positive nuclei, indicating nuclei from cells with comprised cell membrane, and cell cycle analysis for HBEC cells after NPs exposure is shown in **Figure 4**. The results showed 8.1 and 10.6% nuclei from dead cell population following exposure to NiO (25 μg/mL) and etoposide, respectively, compared to 5.4% in control cells. In addition, NiO exposure caused a slight increase in G2/M population (15.9%) and etoposide, a known cell cycle inhibitor, caused significant G2/M arrest (76.2%) compared to control 14.1% (**Figure 4**).

NPs Interference Study

In order to elucidate possible assay interference, additional tests were performed using THP-1 cells. For this test, nuclei from control cells were compared to nuclei spiked with NPs as well as a sample containing only NPs. The results showed only a minor non-significant increase in MN in controls spiked with NPs (0.7%) compared to the control cells (0.5%). In the sample containing only NPs, there was a background observed in FSC vs. SSC dot plots (not shown), however the NPs were not detected in the gated MN and nuclei populations suggesting the reliability of the flow cytometer method (**Figure 5C**). Nevertheless, as different NPs possess different physico-chemical properties it is recommended to use the particle controls in parallel with samples to evaluate the interferences.

Secondary Genotoxicity of NiO NPs (in HBEC Cells After Exposure of dTHP Cells)

From the co-culture setup, dTHP were assessed for cytotoxicity and results indicated there was no notable change in cell viability after 48 h, which might be due to the high cell number used in the study (**Figure 6A**). Further, secondary genotoxicity evaluation in HBEC cells suggested no significant induction of MN in neither NiO nor LPS exposed cells compared to control cells (**Figures 6B,C**).

Secondary Genotoxicity of NiO NPs in HBEC Cells Assessed by Comet Assay

In order to compare the MN formation with DNA strand breaks, comet assay was performed after 24 h exposure of dTHP-1 cells to NiO NPs. In line with the results from 48 h exposure, no significant cytotoxicity was observed compared to control (**Figure 7A**). However, secondary genotoxicity in terms of increase in DNA strand breaks in HBEC cells was observed following dTHP-1 exposure to 25 μg/mL NiO NPs. There was 2.2- and 3.8-fold increase in comet tail intensity for 5 and 25 μg/mL NiO concentrations compared to control (**Figure 7B**). Further, (lipopolysaccharide) LPS treatment did not show any DNA damage in HBEC cells.

Metal Release From Inserts Analyzed by ICP-MS

In order to explore whether Ni (as NPs or ions) added to the insert in the upper compartment could be transferred to the cells in the lower compartment, ICP-MS analysis was performed to analyze Ni content of the media in the lower compartment. This was done both with and without cells (dTHP-1) on the insert. The results showed 1.1 μg/mL Ni in the media when NiO NPs was added without cells. This represents 4.4 wt% transfer of Ni to the lower compartment (data not shown). In presence of dTHP cells, the Ni content of the media 0.44 μg/mL (approx. 1.6 wt%). This suggests that some Ni was transferred (as NPs or ions) even in the presence of cells.

DISCUSSION

The main aim of this study was to establish a flow cytometry protocol for MN analysis that is useful for detecting primary and secondary genotoxicity of NPs. Importantly, possible interferences were also considered. NiO NPs were selected as model NPs due to our previous experience with these NPs using other methods. Our group previously showed the possibility to detect secondary genotoxicity caused by NiO NPs using "conditioned media" and co-cultures of HBEC and THP-1 (Akerlund et al., 2019). In this study, we tried further to understand the secondary genotoxicity of NiO NPs by standardizing a protocol to be used for assessing MN formation detected by flow cytometry. Moreover, this MN flow cytometric protocol was compared with comet assay to explore the secondary genotoxicity in HBEC cells co-cultured with dTHP.

Compared to single *in vitro* experiments, multi-cell models are preferable as they mimic the *in vivo* conditions better and offer an opportunity to detect a broader potential damage caused by NPs (Evans et al., 2019). Only few studies have, however, used such approaches for genotoxicity assessment of NPs or nanomaterials. For instance, a study on different iron oxide NPs evaluated the chromosomal damage by the *in vitro* micronucleus assay, and results indicated that only γ-Fe_2O_3 induced MN formation in lung monocultures. In contrast, immune cell conditioned media and dual cell co-culture approaches indicated that both γ-Fe_2O_3 and Fe_3O_4 NPs were genotoxic towards lung cells due to secondary genotoxicity (Evans et al., 2019). Further, genotoxic effects of few layer graphene evaluated by cytokinesis blocked micronucleus (CBMN) assay revealed significant MN induction in TT1 cells (lung cells) confirmed by both mono and co-culture approaches (Burgum et al., 2021).

To determine the genotoxicity in terms of MN formation, most of the studies use conventional microscopic methods. However, high-throughput techniques are in general getting more attention (Nelson et al., 2017) and MN detection using flow cytometry is gaining more interest. This method has the advantage to gather much information on cytotoxicity, cell cycle analysis, and MN formation in an efficient manner from the same experiments. Further, background from NPs can be minimised using cell free controls in laser-based systems, which might be difficult to interpret in microscopic analysis as NPs at higher concentrations might camouflage the MN population (Vallabani et al., 2014). Our results indicated that the THP-1 cells

appeared more sensitive compared with the HBEC cells. One explanation could be a higher uptake of the particles in THP-1 cells. We did not carefully evaluate the particle internalization in this study (e.g., using TEM imaging), but in a recent study with focus on particles from 3D-printing we noted MN formation in HBEC cells by cobalt nanoparticles (used as positive control) indicating uptake of these nanoparticles (Vallabani et al., 2022).

We and others previously studied MN formation (primary genotoxicity) of various nanoparticles using flow cytometry (Di Bucchianico et al., 2017; Lebedova et al., 2018; Garcia-Rodriguez et al., 2019). Overall, these appear to be in good agreement with the microscopic method and thus, the flow cytometry version has been recommended (Garcia-Rodriges et al., 2019). Also, in previous studies we and others used *in vitro* microflow kit or similar methods; the method is easy to process and rapid in acquiring data (1.0 to 5.0 × 10^4 nuclei per sample) compared to microscopic analysis (Bryce et al., 2008; Vallabani et al., 2014; Vallabani et al., 2019). The detailed protocol published here could be an option or complement to the kit-based method. It also offers the possibility to study cell cycle perturbations as we did for the HBEC cells, (see **Scheme 2**, **Figure 4**). Data showed minimum increase in dead population (EMA +ve) after NiO treatment (25 μg/mL) compared to control. Further, cell cycle alteration was not detected, and there was less G2/M arrest after NPs exposure. In contrast, the positive control "etoposide" a known inducer of double strand breaks triggered a significant G2/M arrest compared to control cells. Similarly, a study in A549 cells exposed to different concentrations of NiO NPs (10, 15, 50, 75, and 100 μg/mL) suggested that cell cycle alterations were only observed at higher concentrations (100 μg/mL) after 48 h (Cambre et al., 2020).

In comparison, comet assay was performed to determine the secondary genotoxicity of NiO NPs in HBEC cells after 24 h exposure. Results expressed increase in DNA damage for both 5 and 25 μg/mL treatment doses; but only the highest concentration 25 μg/mL exhibited a significant increase in tail intensity compared to control. Our earlier study showed a similar genotoxic effect in HBEC cells co-cultured with dTHP. Macrophage exposure with NiO NPs at 50 μg/mL caused a significant DNA damage in HBEC cells after 3 and 24 h (Akerlund et al., 2019). Since a minor part of the Ni (approx. 1.6 wt%) was transferred from the upper compartment with dTHP-1 cells to the lower compartment with HBEC cells, we cannot totally rule out that this affected the DNA breaks formed.

Overall, this study established a flow cytometry protocol for MN analysis that is useful for detecting primary and secondary genotoxicity of NPs. Our results also emphasize the sensitivity of THP-1 cells and thus, these may in general be a good model for assessing MN formation in future studies. Even though our analysis did not find any interference with the NPs and MN detection, it is always important to consider possible NP-assay interferences. Hence, it is also recommended to employ a set of interference controls applied for any nanomaterials and cells used in the study to improve the data reliability (Franz et al., 2020). The present study suggests that NiO NPs did not cause MN formation *via* secondary (inflammatory driven) mechanisms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NV standardized the protocol, performed experiments, analyzed data and drafted the paper; HK designed the project, secured the funding and edited the article.

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Common Considerations for Genotoxicity Assessment of Nanomaterials

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Genotoxicity testing is performed to determine potential hazard of a chemical or agent for direct or indirect DNA interaction. Testing may be a surrogate for assessment of heritable genetic risk or carcinogenic risk. Testing of nanomaterials (NM) for hazard identification is generally understood to require a departure from normal testing procedures found in international standards and guidelines. A critique of the genotoxicity literature in Elespuru et al., 2018, reinforced evidence of problems with genotoxicity assessment of nanomaterials (NM) noted by many previously. A follow-up to the critique of problems (what is wrong) is a series of methods papers in this journal designed to provide practical information on what is appropriate (right) in the performance of genotoxicity assays altered for NM assessment. In this "Common Considerations" paper, general considerations are addressed, including NM characterization, sample preparation, dosing choice, exposure assessment (uptake) and data analysis that are applicable to any NM genotoxicity assessment. Recommended methods for *specific assays* are presented in a series of additional papers in this special issue of the journal devoted to toxicology methods for assessment of nanomaterials: the *In vitro* Micronucleus Assay, TK Mutagenicity assays, and the *In vivo* Comet Assay. In this context, NM are considered generally as insoluble particles or test articles in the nanometer size range that present difficulties in assessment using techniques described in standards such as OECD guidelines.

Keywords: nanomaterials, genotoxicity, methods, mutagenicity, clastogenicity, biocompatibility

INTRODUCTION

Engineered nanomaterials (NM) can have biological effects that differ from those of materials with the same chemical composition, as a result of size, shape, and surface area or surface chemistry. Such differences may include altered biological activity such as uptake, distribution or biological interactions. The small size leads to increased surface area relative to the mass of the particle, which could affect biological disposition and interactions. These same physical and chemical

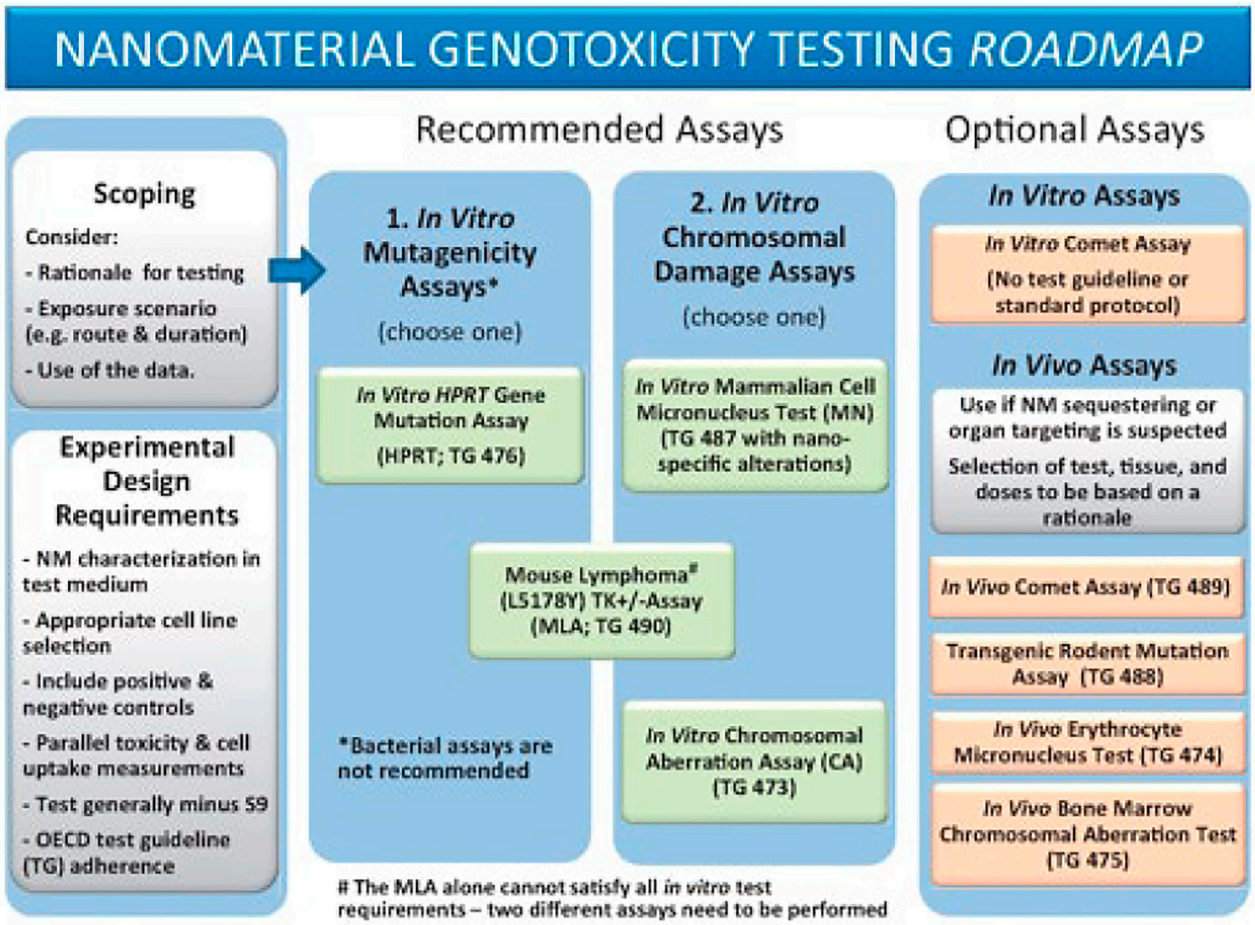

FIGURE 1 | Reproduced from Elespuru et al. (2018), p. 393, with permission from Oxford University Press.

properties may impact the genotoxicity assays designed to assess the potential hazard of NM (Dusinska et al., 2017; ISO, 2017; Elespuru et al., 2018; Faria et al., 2018).

The methods considerations provided in this and accompanying papers are a follow-up to those addressed earlier by Elespuru et al. (2018), in critiques of the issues and problems in the published literature on genotoxicity assessment of NM. The lack of reliable publications related to accurate hazard identification and risk assessment of NM causes problems, especially related to cancer risk assessment.

As noted by others and summarized in Elespuru et al. (2018), some of the problems relate to aspects of the tests that need to be adjusted for assessment of NM, due to interference of nano-sized materials with the test or the endpoint, or lack of uptake of particles into the target cells. Other issues relate to the lack of standard systems, e.g., specific cell lines, used generally for genotoxicity studies, as opposed to myriad cell systems found in the literature that may yield uninterpretable results. Thus, the Common Considerations document and accompanying methods for specific assays are models for genotoxicity testing and assessment of NMs. As noted by others and summarized in Elespuru et al. (2018), bacterial (Ames) reverse mutation assays are not recommended for assessment of NM; thus, a protocol for this assay is not included. This Common Considerations paper consists of a set of issues to be addressed relative to methods and approaches common to the genotoxicity assays, including material characterization, sample preparation, metabolic activation (if needed), dose selection, exposure assessment (e.g., uptake) and data assessment.

The following parameters are considered common considerations for any of the genotoxicity tests recommended in the Toxicological Sciences Roadmap (**Figure 1**) and should accompany the methods on the *In Vitro* Micronucleus Assay, the thymidine kinase (TK)-based mutagenicity assays, and the *In Vivo* Comet Assay.

METHODS CONSIDERATIONS

Integrating Information From Other Tests, Including Animal Assays

Toxicity testing *in vivo* is invaluable for obtaining information on biodistribution, accumulation, and clearance of NM that cannot be assessed using *in vitro* assays. If data from these studies are

available, attention should be paid to the features of these *in vivo* effects, especially regarding tissue or organ sequestering of NM (part of "scoping", **Figure 1**) (Elespuru et al., 2018). Agglomeration or aggregation characteristics of NM in the *in vitro* and *in vivo* tests are also important to consider as they may interfere with the assay or cause unexpected effects. NM are generally not soluble in aqueous media and may be present as suspensions during the test.

Nanomaterial Description

The source of the NM should be provided, i.e., purchased (source) or manufactured/synthesized at the researcher's institution. A physical description of the NM would include chemical composition, structure (size and shape), surface chemistry (where relevant), and an assessment of material or particle diversity, preferably accompanied by a microscopic image. Other features that could be described, if known, include chemical nature of impurities, stability, and capability of the NM to release ions or other moieties.

Nanomaterial Characterization

NM characterization generally includes properties such as chemical composition and physical aspects such as particle size, aggregation and agglomeration characteristics, surface chemistry, surface coating, functionalization, and morphology (shape, surface area, surface topology). Many methods are recommended for NM characterization, based on spectroscopic or imaging technologies (Zhu et al., 2013; Lin et al., 2014; Committee et al., 2021). For example, the primary sizes of NM can be determined using transmission electron microscopy. A certain number of NM should be measured, and the size distribution of the particles and aggregates calculated. However, a common set of methods, many of which depend on specialized instrumentation, has not been established. Methods for NM characterization are provided in references (Zhu et al., 2013; Lin et al., 2014; Dusinska et al., 2017; ISO, 2017; Faria et al., 2018; Committee et al., 2021). Ideally two or more different methods are recommended to measure each parameter in order to minimize the risk of artefacts.

The most important characterization is an assessment of NM properties within biologically representative media. This includes providing an understanding of agglomeration under experimental conditions, material stability and evaluation of the transformation of the material during the experiment, e.g., changes to surface chemistry and/or morphology, and material dissolution. Data comparing the physico-chemical characteristics of the NM in the original supplied form and under experimental conditions (i.e., in medium) are likely to be informative for NM effects in actual use situations.

As noted above, agglomeration and aggregation of particles is an important factor that should be addressed and monitored in sample suspensions before and after testing. Toxicological testing is generally valid for un-agglomerated particles, or as expected in real world use, if agglomeration is expected in real use situations. Since agglomeration is more likely at higher doses, agglomeration should be assessed to assist in choosing the higher doses proposed for the test. Due to their high surface energy, NM may also interact with the testing medium or bind to different substances, including proteins in the test medium or in the *in vivo* environment, possibly resulting in altered biological activity. These factors should be considered if relevant to specific routes of exposure, such as effects in the gastrointestinal tract after oral dosing.

Generally, the dynamic light scattering (DLS) technique can be used to characterize the behavior of the NM. Hydrodynamic size and surface charge can be measured using a Zetasizer or another equivalent instrument. Cell uptake of the particles can be confirmed with microscopic images with or without tags such as metals. Chemical and other analyses can be used to identify NM composition.

Sample Preparation

Describe sample preparation and provide justification for the choice of the suspending medium (vehicle) which should be compatible with the assay used. Due to solubility issues, NM are often present as a dispersion of particles. Information should be provided on handling of the NM, such as sonication of the suspension. Suspensions of the NM test article should be prepared just before use in the assay. Description of NM storage and the potential for change in properties during storage should be considered.

Dose Selection

Dosing and dose-response assessments are critical factors in the safety assessment of NM. In our review (Elespuru et al., 2018) we noted a lack of a rationale for often excessive amounts/doses of NMs used in genotoxicity assays. Excessive doses may create artifacts that are not representative of real use situations, or even mask a real effect (e.g., reverse dose-response curves, where higher doses have less effect than lower doses). Dose selection is still a difficult issue, without consensus, but dose limits for NM are generally considered lower than those in the OECD guidelines. A rationale for dose choices should be provided using experimental or published data. Exposures expected during actual use of the NM are useful for interpretating results, but alone they are not adequate determinants of dosimetry for safety assessment. Toxicological assessments are customarily conducted at higher than actual use doses in order to compensate for uncertainty, as well as to assure detection of a response that may be missed at lower doses. OECD guidelines indicate dosing limits for specific assays; these exposures should be included in the dose-response if they don't interfere with the assay or generate artifactual results. Appropriate dose-spacing to inform NM effects is a critical feature of valid testing. When toxicity is observed, doses should range from non-toxic levels to varying toxicity levels up to a maximum recommended in the OECD guideline for the assay being performed, generally based on cytotoxicity in the test system or the onset of agglomeration or aggregation. The assessment of solubility/dissolution rate, dispersion, aggregation, and agglomeration should be considered for each dose. A total dose-range of 20 to 50-fold, with dose spacing chosen based on preliminary experiments, is recommended for the definitive test. Once a dose range is determined in preliminary experiments, a narrowed set of

doses varying by approximately 2 to 3-fold should be chosen for the definitive test. OECD guidelines may be informative for dose choices for specific tests, but upper exposure limits for NM may be lower, because of agglomeration and other factors (Wills et al., 2017). Dose limits should be justified by experimental data on dose-related agglomeration, aggregation, inflammatory effects (*in vivo*), or potential artifactual results (impacting the assay or test conditions).

Uptake/Exposure

A major consideration for a valid *in vitro* NM genotoxicity test is uptake by the cells to indicate cell exposure. Effects of released ions from NM would qualify as appropriate for targeted analysis.

Some NM physicochemical properties may alter transport of chemical agents into cells. For instance, Ag (silver) ions are transported into bacteria, but nano Ag is not taken up (Butler et al., 2015). This paper also demonstrates multiple methods, including the use of flow cytometry in determining uptake of a NM.

Ideally, information on uptake would be provided for the NM and the cell system under study. If uptake studies are possible, they provide valuable information enhancing genotoxicity data, particularly in the case of a negative test. General principles and methods addressing uptake assessment are provided (Hondow et al., 2011; Kettler et al., 2014; Zhang et al., 2015; Behzadi et al., 2017; Wu et al., 2019). If it is not possible to provide experiments demonstrating NM uptake, dose-response experiments should demonstrate toxicity within acceptable parameters of agglomeration, if not to the limits described in the OECD test guidelines. This provides evidence that the material reached the test system and exposure was effective.

For *in vivo* assays, evidence of distribution to target cells or evidence of released ion effects is necessary for a valid test. Validity of a negative result requires evidence that the NM test article reached the target cells. Acceptance of a positive result requires evidence that the NM exposure did not overwhelm the test system, producing artefactual results. For example, abdominal hemorrhage following a large dose may cause systemic toxicity irrelevant to lower doses. Lack of systemic bioavailability in many cases may be adequate evidence of lack of hazard. However, lack of uptake, and possible false negative results, can result from the use of inappropriate test systems (such as those based on the use of bacteria).

Positive and Negative Controls

Positive controls are designed to demonstrate that the test system is capable of delivering the response or outcome being queried. Although positive NM controls are being sought for several genotoxicity assays, in principle, positive controls do not need to be NM. The most extensive studies of a potential nanoparticle positive control are of WC-Co (Tungsten Carbide Cobalt) by Moche et al. (2015), including studies in gene mutation assays, *in vitro* micronucleus assays and comet assays. Results were significantly positive but somewhat variable. NM genotoxic responses are typically weak. These authors concluded that the mode of action (MOA) was likely via oxidative damage. However, further studies are needed on NM effects. Because positive controls are designed to demonstrate assay integrity, studies with NM test articles are generally performed in assays with standard non-NM positive controls (noted in OECD guidelines for each assay) that produce robust responses in the assays.

Negative controls are the solvent vehicle in which the NM is suspended. Typical negative controls are compatible with the biological test system used, and include water, saline, or cell culture medium. If non-standard vehicles are used, it should be demonstrated in preliminary experiments that the vehicle in use does not affect the test system or outcome of testing.

If a NM is expected of interfering with the assay endpoint measurement or biological response, the positive control could be run with and without the NM to determine an inhibitory effect or interference in the positive control outcome.

Metabolic Activation

Many carcinogens and genotoxins require metabolic activation to reactive forms that cause diverse genotoxic effects. Thus, for valid safety assessment, genotoxicity testing generally includes sets of tests in the presence and in the absence of a metabolic activating system. Whereas *in vivo* systems contain inherent metabolic activating capability, *in vitro* assays require the addition of an activating system. The standard *in vitro* metabolic activation system consists of a 9,000 × g liver homogenate (S9) from rats treated with phenobarbital/β-naphthoflavone (or other validated inducers), plus cofactors. Chemicals may become more or less reactive/active in the presence of the metabolic activation system. However, few if any NM are known to require metabolic activation for generation of a positive genotoxicity response. In order to save animals, materials and time, we recommend that most NM do not need to be tested with S9 metabolic activation mix, including e.g., metal or polymer NM. However, if metabolic activation is indicated, the standard recipe mix and alternative resources are provided here.

The final concentrations of the co-factors in the S9 mix consists of:

- 5 mM glucose 6-phosphate,
- 4 mM nicotine-adenine dinucleotide phosphate (NADP)
- 8 mM MgCl2
- 33 mM KCl in a 100 mM phosphate buffer at pH 7.4.

S9 fraction and cofactor mixes are available commercially or may be prepared in-house. The freshly thawed (and kept on ice) S9 preparation is mixed with the cofactor pool in defined amounts to result in e.g., 10% S9 and 1X cofactors. This is the S9 mix, which is added to test systems in defined amounts, e.g., into mammalian cell assays at 10% resulting in a final concentration of 1% S9. See Maron and Ames (Maron and Ames, 1983).

RESULTS: EVALUATION AND INTERPRETATION OF NM TEST RESULTS

A test result is considered clearly positive or negative based on the following criteria (**Table 1**).

Both criteria should be met to consider a result clearly positive or negative. There are cases where it is not possible to determine a

TABLE 1 | Criteria for positive or negative result.

	Clearly positive	Clearly negative
Criteria	1. At least one of the test groups* exhibits a statistically significant increase in the assay endpoint compared to the concurrent negative control 2. Any of the results are outside the distribution of the historical negative control data (e.g., 95% control limits)	1. None of the test groups* exhibit a statistically significant increase in the assay endpoint compared to the concurrent negative control 2. All results are inside the distribution of the historical negative control data (e.g., 95% control limits)

**Test item, test article or test group: the solution, suspension, or other preparation of the NM added to the test; a test group would be one dose sample among several of the samples in the assay.*
"Historical control data" refers to accumulated data from previous experiments.

clearly positive or a negative result. Then, a repeat experiment is recommended with a modified study design to clarify results, for example, with more closely spaced dose levels in the optimum dose range, and/or increased numbers of cultures per dose). Genotoxicity test guidelines typically recommend a dose-response study as a criterion for a clearly positive result. In the case of NM, a dose-response may not be observed. Higher doses where agglomeration occurs may decrease cell uptake and thus lead to an abnormal dose response (Wills et al., 2017). Therefore, a dose-response is not required for a clearly positive result when testing NM. However, dose-response and reproducibility information should be included in the assessment, along with information on NM properties, including e.g., changes in agglomeration as a function of dose. In case a clearly positive or negative result cannot be determined after a repeat of the experiment, results may be considered *equivocal*.

DISCUSSION

Results should be discussed in terms of technical analysis of the properties of the NM and its characteristics that are relevant to the results. For example, what is the dynamic range of the induced effect, if a positive result is observed? How might the result inform the mode of action, e.g. as a direct or an indirect genotoxic effect? If bioavailability was not achieved in conjunction with a negative result, this should be discussed. What is the impact of this result on hazard consideration of the NM?

RECOMMENDATIONS GUIDELINE

As noted, we don't think specific recommendations are appropriate to address NM issues at this time, but the following general recommendations are provided for consideration.

- Scoping: what is the purpose of the testing/assessment?
  - General hazard identification
  - Specific question or focus
- Test selection (from the Genotoxicity test battery adapted to NM, Roadmap (**Figure 1**)
  - Gene mutation: TK mutation assays: Mouse Lymphoma (MLA), or TK6
  - Clastogenicity (large scale DNA damage): *in vitro* Micronucleus Assay, or MLA
  - *In vivo* assessment: Comet Assay (DNA strand breaks)
- NM assessment
  - Characterization (in the test medium if possible)
    - Choice of assessments: size, shape, distribution, uniformity, representative photo
    - Choice of instrumental measurements
  - Sample preparation
    - Vehicle selection: NM ideally in suspension in bio-compatible vehicle
    - Potential agglomeration?
    - Sonication?
  - Dose selection
    - Dose-range finding study
    - Dose choice
      - Meets assay requirements (OECD guideline suggested limits may not be applicable)
      - Does not interfere with the assay
      - NM can be separated from the test system after exposure time
  - Exposure assessment
    - ADME: distribution in animals (if info is available for consideration)
    - Uptake into cells
    - Fate of particles
    - Fate of marker such as ion or element
  - Negative and position controls
  - Data analysis

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Data are referred to in the references.

AUTHOR CONTRIBUTIONS

Conceptualization: RE; Writing—Original Draft preparation: RE; Methodology, Data Curation, Writing—Review and Editing, Resources: RE, RC, SD, AC, MD, SP, MM and CC

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METHODS
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In vivo Mammalian Alkaline Comet Assay: Method Adapted for Genotoxicity Assessment of Nanomaterials

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The *in vivo* Comet assay measures the generation of DNA strand breaks under conditions in which the DNA will unwind and migrate to the anode in an electrophoresis assay, producing comet-like figures. Measurements are on single cells, which allows the sampling of a diversity of cells and tissues for DNA damaging effects. The Comet assay is the most common *in vivo* method for genotoxicity assessment of nanomaterials (NM). The Method outlined here includes a recommended step-by-step approach, consistent with OECD 489, taking into consideration the issues impacting assessment of NM, including choice of cells or systems, handling of NM test articles, dose determination, assay methods and data assessment. This method is designed to be used along with the accompanying "Common Considerations" paper, which discusses issues common to any genotoxicity assay using NM as a test article.

Keywords: nanomaterial, comet assay, single cell gel assay, genotoxcicity, DNA damage, hazard identification

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INTRODUCTION

The methods found in this issue of *Frontiers in Toxicology* are devoted to Nanomaterials (NM) assessment. Four papers in the series are a follow-up to the analysis and critique of the literature on genotoxicity assessment of NMs by an international group working together via the GTTC (Genetic Toxicology Testing Committees) of the Health and Environmental Sciences Institute (HESI) (Elespuru et al., 2018). Besides this method for *in vivo* assessment of genotoxicity, a paper describing "Common Considerations" as well as two other methods for *in vitro* mammalian mutagenicity or clastogenicity are described in separate papers.

Although *in vitro* genotoxicity assays may be sufficient for assessment of genotoxicity in many contexts, *in vivo* assays may be uniquely valuable in assessing distribution or sequestration of NM, because of physical characteristics, that would not be detected otherwise. As noted in Elespuru et al. (2018), *in vivo* assays may be recommended if other data or circumstances indicate a NM distribution consistent with a sequestration or specific targeting. *In vivo* assays are not recommended as a primary screen for NM effects and should be justified. To fulfil reduction,

refinement, and replacement (3 R's) animal welfare requirements, this protocol can be integrated with other toxicological endpoints in a single animal study.

The *in vivo* alkaline comet (single cell gel electrophoresis) assay (hereafter called comet assay) is used to identify substances that cause DNA interactions that are generally related to DNA strand breakage. Cells or nuclei isolated from tissues of animals that have been exposed to the test article are embedded in agarose and lysed to form nucleoids. Electrophoresis causes DNA with breaks to extend towards the anode, giving the appearance of a comet under fluorescence microscopy; the relative intensity of DNA in the comet tail reflects the break frequency. The analysis does not allow for the discrimination of the origin of the strand break (e.g., direct break, intermediary DNA break introduced by repair mechanism, or indirect break resulting from inhibition of other bioprocess), or for the detection of DNA cross-links. See OECD Test Guideline (TG) 489 (OECD, 2016; Brunborg and Collins, 2020) for additional information.

For mechanistic studies, an additional step can be added to the standard comet assay - incubation with a DNA repair enzyme [e.g., 8-Oxoguanine glycosylase (OGG1) or formamidopyrimidine-DNA glycosylase (Fpg)] to detect oxidized DNA bases (Collins et al., 2017) [It is noted that the modified comet assay to detect DNA oxidation damage is not yet validated and the current OECD TG 489 (OECD, 2016) for the standard comet assay does not provide recommendations for the modified comet assay].

Although an *in vitro* comet assay could be informative, a universally accepted protocol or an OECD guideline for an *in vitro* version of the comet assay does not currently exist. For this reason, the *in vitro* comet assay is not recommended and not considered in this series of papers by the GTTC group.

TEST SYSTEM

Animal Strains

Various common laboratory strains of rodents (e.g., Sprague Dawley, Wistar Han, or F344 rats; CD1, BALB/c, or ICR mice) can be used for this assay. Animals should be 6–10 weeks old at the start of the treatment and within normal weight for their age (animal variation should not exceed 20% of the mean weight of each sex).

Animal Housing and Feeding

The animals should have a minimally invasive unique identifier and be randomly assigned to treatment groups. Animals should be socially housed (up to four same sex animals per cage except if aggressive behavior is noted) in solid floor cages with hardwood chip bedding and micro-isolator bonnets. Animals should also be provided with items such as a hiding device and a chewing object. Conventional laboratory diets along with drinking water can be given *ad libitum* throughout the course of the study. The environment of the animal rooms is set to maintain a 12 h light cycle, temperature of 22 ± 3°C, relative humidity of 30–70%, and air changes of 10–15/h. The rats are provided standard pelleted food and purified water (e.g., distilled/deionized water) ad libitum (OECD, 2016). The care of animals and all animal experimental procedures will be performed in accordance with a study protocol approved by the Institutional Animal Care and Use Committee.

Preparation of NM for Testing

NM characterization is generally required for data interpretation and publication. The NM should be prepared for testing, e.g., by sonication of particles and suspended in a non-toxic vehicle compatible with the test system (e.g., water for injection, physiological saline, ethanol, methylcellulose solution [See Elespuru et al., 2022]).

Since fluorescent lighting can induce oxidative damage, all procedures for the assay should be performed to protect the test articles from light exposure. If other than well-known vehicles for administration of NM are used, reference data demonstrating their compatibility with the test system should be provided. In the absence of previous data demonstrating no effect on comet induction, an initial study should be performed to qualify the vehicle.

PRELIMINARY CONSIDERATIONS

Route of Administration

The route of administration may cover the intended or reasonably expected route of exposure to humans, if feasible. If more than one route of exposure is expected, then a rationale should be presented to select the route leading to higher exposure or that is expected to be the most sensitive. The gastrointestinal tract contains a layer of mucous which functions to prevent particles from contacting Payer's patches and other entry portals. Gavage volumes should be minimized to avoid using large volumes of liquid vehicles which may "wash" away this protective coating.

Proof of NP or NM Exposure and Cellular Uptake

If ADME (absorption, distribution, metabolism, elimination) studies on particle distribution are not undertaken, uptake of the NP into the cells analyzed should be assessed and the location of particles within the cells (i.e., nucleus or cytoplasm) determined if feasible. See the Common Considerations paper for additional information on uptake of NMs and methods for determination of exposure. In some cases, comet assay results may be positive in the absence of cellular uptake. This could reflect the consequence of artifacts such as tissue inflammation-related reactive oxygen species, toxicologically valid events such as breakdown of the material in the test environment, or the release of diffusible substances.

Proof of cellular uptake or target organ exposure is recommended for hazard identification. In cases where experimental data demonstrate that cellular uptake does not occur under the condition of testing, a negative test result may be consistent with a lack of exposure. A demonstration of

exposure, lack of exposure, or systemic distribution is recommended for an evaluation of negative results.

Dose-Range Determination

If a preliminary range-finding study is performed to support dose selection, it should be performed under similar conditions to those intended for the main study with the same species, strain, sex, test article preparation, route of administration, and treatment regimen. The study should aim to identify the maximum tolerated dose (MTD) without evidence of study-limiting toxicity for the duration of the study period (e.g., no death or evidence of pain, suffering or distress of the animals; no suppression of body weight gain, hematopoietic system toxicity, or increased inflammatory biomarkers). Inflammation is an important confounding factor for the comet assay. Thus, it is advisable to consider the impact of dose regimens that could lead to high levels of inflammation in the target tissue. Animals should be observed hourly for the first 4 h following each administration of test article. If the test article does not elicit toxicity under the conditions planned for the main experiment, the highest dose may be based on evidence of viscosity, NM dispersibility, aggregation or agglomeration. The study design and options for selection of the maximum dose can be found in the literature (Delmaar et al., 2015; Faria et al., 2018).

EXPERIMENTAL METHOD [REFER ALSO TO OECD 489, 2016]

Controls

The comet assay is conducted with groups including vehicle control, at least three dose levels of test article, and a positive control. The selection of a positive control for the study should be based on demonstrating clear positive comet responses in the tissues of interest. Methyl methanesulfonate (CAS RN 66-27-3) is a widely used positive control as it has produced DNA strand breaks in all rodent tissues that have been studied. Other positive controls include ethyl nitrosourea (CAS RN 759-73-9), ethyl methanesulfonate (CAS RN 62-50-0), N-methyl-N′-nitro-N-nitrosoguanidine (CAS RN 70-25-7), 1,2-dimethylhydrazine 2HCl (CAS RN 306-37-6) and methylnitrosurea (CAS RN 684-93-5). Samples of positive target tissues for the respective positive controls can be found in the OECD TG 489. Positive controls other than these should only be selected if scientifically justified. Currently there is no NM specific positive control that has its response clearly defined. It is not necessary to administer concurrent positive control substances by the same route as the test article.

Each experimental group should have at least five animals (per sex, if both sexes are used) with no fewer than three animals acceptable for the positive control.

The route of administration to simulate human exposure may be selected from among the following options: dietary, drinking water, topical, subcutaneous, intravenous, oral (by gavage), inhalation, or implantation. Intraperitoneal injections of test article should be avoided unless specifically justified. The size of test animals should be considered when determining the maximum volume of liquid that can be administered in accordance with animal welfare legislation.

Treatment Schedule and Dosing

Animals should be treated daily over a duration of two or more days and samples should be collected at 2–6 h after the last treatment, or at the time of maximum plasma concentration (if known). A longer treatment schedule may be used to allow the incorporation of the comet assay endpoint into other toxicology repeated dose assays, but one additional dose may need to be considered to satisfy the requirement for the collection of samples at 2–6 h after the last treatment.

Observations

Clinical observations about the health of the animals need to be recorded at least once a day, considering the peak period of anticipated effects after dosing. Animals should be observed at least twice a day and at the end of the exposure period for morbidity and mortality. For longer duration studies, animals should be weighed at least once a week. If the test article is administered via feeding and drinking, then water and food consumption should be recorded. Animals showing signs of excessive toxicity should be euthanized prior to completion of the study and not used for comet analysis.

Tissue Collection

Tissues considered for study should include the site of contact such as stomach/duodenum/jejunum for oral exposures, an organ representing systemic distribution such as liver, and an organ such as kidney where bioaccumulation may occur.

Animals should be sacrificed by procedures accepted by effective animal welfare regulation at the appropriate time after the last treatment. The selected tissue should be dissected, with resulting pieces used for comet preparation and for potential histopathology examination. Tissue for comet evaluation should be rinsed with 0.9% sterile saline, exsanguinated, and placed in rinsing buffer (see solution recipes at the end), and kept ice-cold until processed.

Specimen Preparation

For all animals, soft tissues (e.g., liver, lung) are minced in cold homogenizing buffer (see recipes) to create a single cell suspension. Blood and bone marrow can be applied to the slide directly. For hard tissues (e.g., glandular stomach, duodenum), the epithelial cells are gently scraped into cold homogenizing buffer prior to passing the released cells through filtration, such as a sieve or mesh, to create a single cell suspension. Since UVA radiation from fluorescent lighting can induce oxidative damage, samples should be harvested in an environment protected from light. The samples are stored ice-cold until slide preparation.

Preparation of Slides

The cell suspension is mixed with 1% low melting point agarose in phosphate-buffered saline (PBS) at 37°C to a final concentration of approximately 0.8%, and drops are placed on the coated slide. There are different formats ranging from 2–20 gel drops per slide. At least three gels per tissue per animal are prepared. Preferably, gels from each sample are set on different slides. Once gels are solidified, the slides are immersed in complete lysis solution in a light-proof box and placed at 4°C, until the electrophoresis step. Slides should be immersed in lysis solution for at least 1 h but can be stored in this solution at 4°C for up to 3 days [Formats allowing a higher

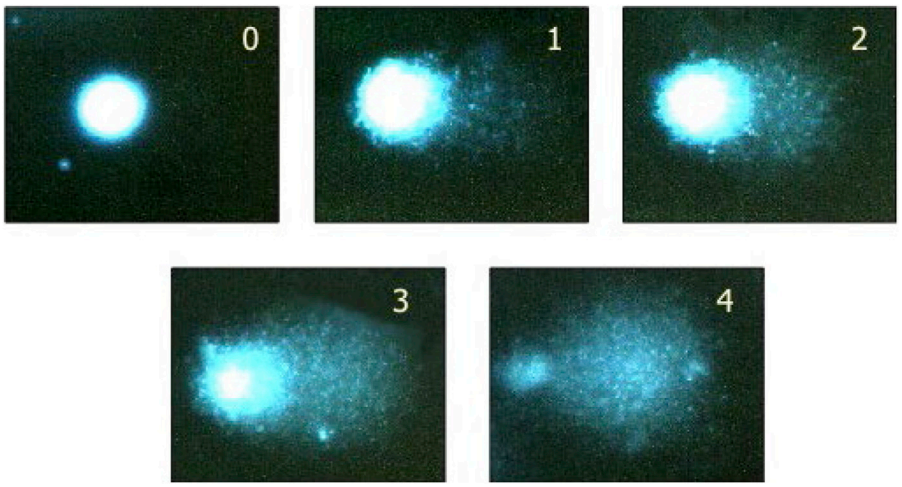

FIGURE 1 | Visual scoring - an acceptable option if image analysis is not available. By eye, it is possible to classify comets into five categories, corresponding to these typical images. Examining 100 comets in this way, and giving scores corresponding to the classes (0, 1, 2, 3, 4), the total score will be between 0 and 400, roughly equivalent to 0–100% tail DNA. See Collins, 2004 (Collins, 2004) for more details, including a direct comparison of visual scoring with computer image analysis (Note: a class 4 comet would commonly be referred to as a 'hedgehog comet'.) While visual scoring is possible, automated scoring is recommended, to avoid bias in data interpretation. Reprinted from Collins, 2004, by permission of Springer Nature.

throughput such as CometChip assay have been devised; they are not covered in this protocol.]

Slide Analysis

Comets are scored quantitatively by an automated or semi-automated image-analysis system (e.g., Comet Assay IV) or by visual scoring (**Figure 1**). The slides are encoded to minimize potential operator bias. The slides are stained with an appropriate fluorescent stain (e.g., Propidium iodide, SYBR Gold, Green I.) and the comets (nucleoids) visualized using a fluorescence microscope at ×200 magnification.

Initially, gels are examined visually for any evidence of overt toxicity, e.g., an increase in background debris and/or an increase in the incidence of excessively damaged nucleoids (e.g., 'hedgehogs'). These nucleoids cannot be quantified by image analysis, but their frequency should be recorded, along with nucleoids that have unusual staining artifacts or comets with non-spherical heads. Where practical, at least 150 nucleoids (excluding hedgehogs) should be analyzed per tissue per animal (e.g., 50 nucleoids on each of three gels). Several representative areas of the slide should be chosen to avoid bias; however, scoring at the edge of the slide should be avoided. Nucleoids should be scored for % DNA in tail (aka % tail intensity) (Burlinson et al., 2007; OECD, 2016).

Expression of Results and Statistical Anaysis

According to consensus, the % tail intensity values should be presented as:

- The median % tail DNA for each slide and average for each animal.
- The mean of these median % tail DNA values and standard deviation of each group

The numerical data corresponding to % tail DNA are statistically evaluated in a tiered approach using two datasets. The first dataset includes the negative control group and the positive control group, to determine the validity of the assay. The second dataset includes the negative control group and the test article groups to determine the genotoxicity of the test article.

Detailed statistical approaches can be found in, (Lovell and Omori, 2008; Bright et al., 2011). Evaluation and interpretation of the results are described in the Common Considerations paper.

Solutions

- Rinsing buffer for tissues: Hanks Balanced Salt Solution, containing 20 mM EDTA and 10% v/v dimethyl sulfoxide, pH 7.4,
- Homogenizing buffer for tissues: Hanks Balanced Salt Solution, with calcium, with magnesium, without phenol red, containing 20 mM EDTA and 10% v/v dimethyl sulfoxide, pH 7.4.
- Lysis solution: 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10 (with NaOH); 1% Triton X-100 added just before use.
- Electrophoresis solution: 0.3 M NaOH, 1 mM EDTA, pH > 13.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the first author.

AUTHOR CONTRIBUTIONS

Conceptualization: RE and RC; Writing—Original Draft preparation: RC; Methodology: RC, MD, AC, MR, SP, and MM; Writing—Review and Editing, Resources: RE, RC, MD, AC, MR, SP, and MM.

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METHODS
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Thymidine Kinase$^{+/-}$ Mammalian Cell Mutagenicity Assays for Assessment of Nanomaterials

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The methods outlined here are part of a series of papers designed specifically for genotoxicity assessment of nanomaterials (NM). Common Considerations such as NM characterization, sample preparation and dose selection, relevant to all genotoxicity assays, are found in an accompanying paper. The present paper describes methods for evaluation of mutagenicity in the mammalian (mouse) *thymidine kinase* (*Tk*) gene occurring in L5178Y mouse lymphoma (ML) cells and in the designated *TK* gene in human lymphoblastoid TK6 cells. Mutations change the functional genotype from TK$^{+/-}$ to TK$^{-/-}$, detectable as cells surviving on media selective for the lack of thymidine kinase (TK) function. Unlike cells with TK enzyme function, the TK$^{-/-}$ cells are unable to integrate the toxic selection agent, allowing these cells to survive as rare mutant colonies. The ML assay has been shown to detect a broad spectrum of genetic damage, including both small scale (point) mutations and chromosomal alterations. This assay is a widely used mammalian cell gene mutation assay for regulatory purposes and is included in the core battery of genotoxicity tests for regulatory decision-making. The TK6 assay is an assay using a human cell line derived similarly via mutagenic manipulations and optimal selection. Details are provided on the materials required, cell culture methods, selection of test chemical concentrations, cytotoxicity, treatment time, mutation expression, cloning, and data calculation and interpretation. The methods describe the microwell plate version of the assays without metabolic activation.

Keywords: nanomaterials, mouse lymphoma, TK6, mammalian mutagenicity, mutagenesis

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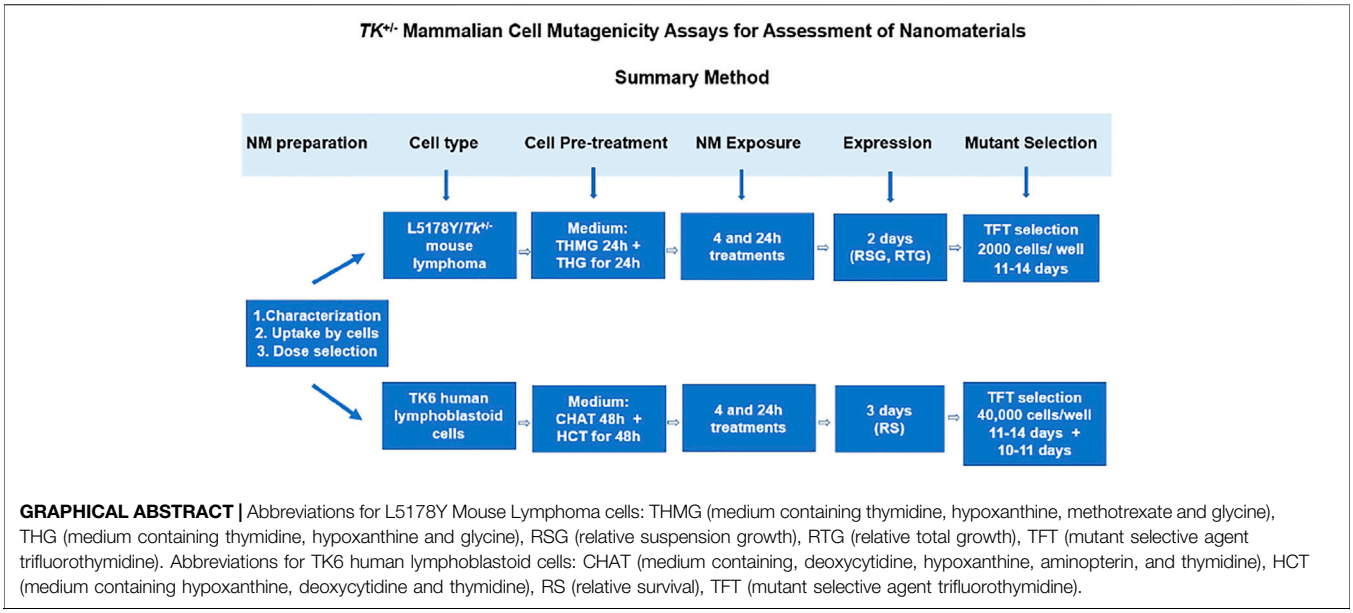
1 INTRODUCTION

The methods found in the Nanotechnology Section of *Frontiers in Toxicology* are a follow-up to the analysis and critique of the literature on genotoxicity assessment of nanomaterials (NM) by an international group working together via the Genetic Toxicology Testing Committees (GTTC) of the Health and Environmental Sciences Institute (Elespuru et al., 2018). The mammalian TK mutagenicity assays described here use the *TK (thymidine kinase)* gene as a target for mutational analysis. OECD Test Guideline (TG) 490 describes parameters for performing the assays (OECD 490, 2016), but the TG does not address methods specific to valid assessment of NM.

The Mouse Lymphoma Assay (MLA), using the heterozygous rodent L5178Y mouse lymphoma cells, is widely used in the genotoxicity test battery because of its capability of detecting both small-scale mutational damage and large-scale chromosomal alterations. Large portions of the gene may be



GRAPHICAL ABSTRACT | Abbreviations for L5178Y Mouse Lymphoma cells: THMG (medium containing thymidine, hypoxanthine, methotrexate and glycine), THG (medium containing thymidine, hypoxanthine and glycine), RSG (relative suspension growth), RTG (relative total growth), TFT (mutant selective agent trifluorothymidine). Abbreviations for TK6 human lymphoblastoid cells: CHAT (medium containing, deoxycytidine, hypoxanthine, aminopterin, and thymidine), HCT (medium containing hypoxanthine, deoxycytidine and thymidine), RS (relative survival), TFT (mutant selective agent trifluorothymidine).

lost without compromising viability, thus allowing the detection of a broad spectrum of genetic alterations (Clive and Spector, 1975; Applegate et al., 1990). The human lymphoblastoid TK6 cells (Skopek et al., 1978; Liber and Thilly, 1982) were developed in the same time frame as the MLA and are considered useful because of the human origin of the cells, which are P53 proficient. However, these cells originated from a person with a hereditary genetic disease and were manipulated by mutagen treatment and selection for their growth capability (Skopek et al., 1978). These cells have come into general use relatively recently. The two cell types are functionally similar as targets for mutation induction and useful as laboratory models for mammalian mutagenesis. (Note that genetic nomenclature is different for human and rodent cells; thus, the differences used here: *Tk* for the mouse gene, *Tk* for the human gene, T_k for the thymidine kinase protein, and *TK* generally referring to genes in both assays).

The assays detect a broad spectrum of genetic damage due to the nature and autosomal location of the TK gene. Two distinct phenotypic classes of TK mutants are generated in these assays, the normal growing and slow growing mutants that are recognized as large colony and small colony mutants, respectively, in the MLA. They are early appearing and late appearing colony mutants in the TK6 assay. Slow growing mutants of both cell types have acquired genetic damage that involves putative growth-regulating gene(s) near the TK locus, resulting in prolonged doubling times and the formation of late appearing or small colonies. More recent studies have demonstrated the molecular nature of mutations in both assays (Hakulinen et al., 2011; Guo et al., 2018). Other results indicate that the assays are sensitive enough to detect mutagenicity of NM (Mei et al., 2012; Elespuru et al., 2018; Demir et al., 2020).

The methods presented in this paper are written specifically for mutagenicity evaluation of NM, providing details of the materials required, cell culture methods, selection of test chemical concentrations, cytotoxicity, treatment times, mutation fixing, cloning, and data calculation and interpretation. The detailed protocols describe the microwell plate version of the assays without metabolic activation. The methods are meant to be used together with the accompanying paper on Common Considerations for Genotoxicity Assessment of Nanomaterials (*Front. Toxicol. doi: 10.3389/ftox.2022.859122*). NM issues related specifically to this assay include dosimetry (toxicity, agglomeration), separation of the NM from the suspension cells, and effects on mutant expression that may require extended incubation times.

2 TEST SYSTEMS

2.1 Cells

Cell types: L5178Y/$Tk^{+/-}$ −3.7.2°C subline, derived from a mouse lymphoma (Clive and Spector, 1975), and TK6, a lymphoblastoid cell line derived from a human genetic disease source (Skopek et al., 1978). Both cell types are grown in suspension culture and maintained in exponential growth for the assay. See (Lorge et al., 2016) for additional information on methods for handling cells.

2.2 Media

- The basic medium (F_{0P}) consists of RPMI 1640 medium supplemented with 100 unit/mL penicillin, 100 mg/ml streptomycin, and 200 mg/ml sodium pyruvate.
- Treatment medium (F_{5P}) contains 5% (v/v) heat-inactivated horse serum or heat-inactivated fetal calf serum in F_{0P}. Note that if the serum is not purchased as heat inactivated serum, it requires heat-inactivation at 56°C for 30 min before use.
- Growth medium (F_{10P}) contains 10% (v/v) serum added to F_{0P}.
- Cloning medium (F_{20P}) contains 20% (v/v) serum added to F_{0P}.
- THMG stock medium (100x) consists of F_{0P} supplemented with 300 mg/ml thymidine, 500 mg/ml

hypoxanthine, 10 mg/ml methotrexate and 750 mg/ml glycine.
- THG stock medium (100x) contains the same components as THMG stock medium without methotrexate.
- CHAT medium contains F_{10P} supplemented with 10 μM 2′-deoxycytidine, 200 μM hypoxanthine, 0.1 μM aminopterin, and 17.5 μM thymidine.
- HCT medium (CHAT without aminopterin)
- TFT mutant selection medium contains 100 μg/ml trifluorothymidine in saline

Media are filter-sterilized and stored at 4°C wrapped in foil to protect from light. THMG, THG, CHAT and HCT media can be stored at −20°C. The media are warmed to room temperature before use.

2.3 Maintenance of Cells

The cultures are grown in polycarbonate tissue culture flasks and placed in a 95% humidified incubator with 5% CO_2-in- air at 37°C. For a valid assay the cells need to be maintained in log phase; doubling times are 9–10 h for L5178Y and 11–12 h for TK6. The cultures are routinely diluted with fresh F_{10P} medium each day to 2×10^5 cells/mL. For longer periods, the cells can be diluted to 7×10^3 cells/mL Doubling times must be carefully monitored because cultures showing prolonged doubling times should not be used for experiments. Cells can be cryopreserved in liquid N_2 using F_{20P} containing 5% dimethyl sulfoxide (DMSO).

2.4 Cleansing Cell Cultures

An essential requirement is elimination of pre-existing *TK*$^{-/-}$ cells that would impact the mutation levels in the negative controls in the test. Cleansing is carried out during the week preceding an assay or prior to freezing many vials for storage in liquid N_2. Note that the cells grow at longer doubling times during cleansing. The cells should not be exposed to test chemicals until they have completely recovered from cleansing, usually after 24 h. Cleansed cells can be cryopreserved at a density of 5×10^6 cells/mL/tube in freezing medium (e.g., 10% DMSO in cell culture medium with serum). New cultures for assays may be started directly from the cryopreserved cleansed stocks, after centrifuging and re-suspended in fresh medium.

To cleanse L5178Y: Cells are treated sequentially with THMG and then THG, as follows: 0.5 ml of THMG (100X stock) is added to 50 ml of cell culture at 2×10^5 cells/mL in F_{10P}. The cells are incubated at 37°C for 24 h in this medium. After counting (the cell density should be ~1.0×10^6 cells/mL), cells are centrifuged at 200 x *g* for 10 min and the pellet is resuspended at a concentration of 2×10^5 cells/mL in 1% THG medium (F_{10P} medium containing 1% THG stock) and incubated for another 24 h.

To cleanse TK6: Cells are incubated in CHAT medium for 48 h and then transferred into HCT medium for 48 h to kill *TK*$^{-/-}$ cells before starting the *TK* assay.

3 CHARACTERIZATION OF NM

Refer to the Common Considerations paper for this information [*Front. Toxicol. doi: 10.3389/ftox.2022.859122*].

4 PREPARATION OF NM FOR TESTING

Most NM are not soluble in aqueous solutions. Make sure solutions are made fresh right before the experiment. A test NM stock solution is prepared by dispersion of the NM in sterilized deionized H_2O or another suitable aqueous solvent by vortexing and then sonicating to ensure a uniform suspension of the NM. Then the stock solutions can be diluted to different concentrations with treatment medium. Usually, NM are not sterilized prior to use because of potential alterations to the material, but all other techniques should be performed under sterile conditions.

5 PRELIMINARY EXPERIMENTS

5.1 Positive and Negative Controls

To ensure that an assay is valid, positive controls and negative controls are included in each experiment. Chemicals commonly used as positive controls for test of any agent in TK mutagenicity assays include methylmethanesulphonate (MMS) at 10–20 mg/ml and 4-nitroquinoline 1-oxide (NQO) at 0.05–0.1 mg/ml. NQO can be prepared in DMSO as a 100-fold concentrated stock solution and stored as frozen aliquots at −80°C**.** However, MMS 100X stocks should be freshly prepared with physiological saline. Reagent solutions should be protected from light. If a solvent other than saline or F_{0P} (culture medium minus serum) is used, the negative control should receive a volume of the solvent equivalent to the highest amount used for a treated culture without exceeding a final volume of 1%. A NM-type positive control, such as Tungsten carbide-cobalt (WC-Co), would be useful; however, validation studies have not been carried out, and widespread use of this or other potential NM positive controls have not occurred. Consensus has not been found yet for a positive NM control for this (or any other genotoxicity) assay. The general requirement that a positive control generate a strong response may prevent the use of WC-Co and other NM as positive controls, since their responses generally are relatively weak. Thus, genotoxicity assessment of NM is carried out using common positive controls for each assay.

5.2 Determination of Exposure Concentrations

If the cytotoxicity of the test NM is unknown in this test system, a preliminary experiment should be performed to define the cytotoxic concentration range. The test NM is suspended in a suitable solvent such as saline, DMSO, or F_{0P} (cell culture medium with no serum) at appropriate concentrations of stock solutions. Stock solutions are created that can be diluted into the test system to generate the desired concentration. Sets of stock solutions are prepared so that the same dilution into the test system (e.g., 1:10 or 1:100) is made for each concentration. If possible, aqueous vehicles compatible with the cell systems should be chosen. Appropriate amounts of stock solutions are added to the cells in suspension in treatment medium to create

the desired final concentrations for each data point in the experiment. The volume added should not exceed 1% (i.e., at least 1:100 dilution) when DMSO or other non-aqueous solvents are used. Such dilutions are necessary to avoid toxic effects of non-aqueous solvents on the test cells. The final volume of solvent or vehicle should be the same in all cultures.

The exposure concentrations of a test NM are selected according to target toxicity ranges, with a maximum allowable cytotoxicity of 10% growth relative to the negative control [see (OECD 490, 2016)]. The exposure concentration selected is also dependent on agglomeration status of the test article, which may signal an upper limit of exposure (see the Common Considerations paper). Generally, one or more preliminary experiments using half-log dilutions may be useful to aid in determining the concentrations used in the definitive assay. Duplicate (or triplicate) cultures are used for the negative/solvent control. See the Common Considerations paper for additional information on selection of concentrations for study (*Front. Toxicol. doi: 10.3389/ftox.2022.859122*).

6 DEFINITIVE ASSAYS

6.1 Chemical Treatment

Selection of concentrations for test may have been informed by a preliminary experiment determining reasonable limits of high and low concentrations selected for assessment. In that case, 4-5 useful concentrations with a relatively narrow concentration range (2 or 3-fold) between exposures may be selected. If a preliminary concentration-response assessment has not been made, then additional test concentrations should be used as a better likelihood of finding an informative dose range. Test concentrations ideally range between non-toxic and moderately toxic (90% survival loss). With many NM it may not be feasible to test into a moderately toxic zone, due to agglomeration or other effects.

The cells should always be maintained in logarithmic growth before cell treatment. For treatment, the test NM solution or suspension, positive control chemicals, or control vehicle are added into 50-ml sterile disposable centrifuge tubes containing 6×10^6 cells for L5178Y or 10^6 cells for TK6 in 10 ml of F_{5P} (cell culture medium with 5% serum). After gentle mixing, cultures are placed in a CO_2 incubator at 37°C for 4 h. A 24 h treatment should be conducted in parallel, or subsequently if the 4 h treatment is negative. For the 24 h treatment, the cell density should be adjusted to 2×10^5 cells/mL to allow for additional growth over the longer incubation period. Cultures may be placed on a rocker platform during treatment to prevent the NM from settling out. After incubation, the cells are centrifuged at 200 x *g* for 10 min and the supernatant is discarded. Each culture is then washed twice with F_{0P}, to remove the NM test article, by centrifuging and resuspending the cells in fresh medium. Notation should be made of visible remaining NM, and a third wash instituted if necessary. After the final centrifugation, the cell pellet is resuspended in 20 ml of fresh F_{10P} (cell culture medium with 10% serum) at a concentration of 3×10^5 cells/mL.

6.2 Expression Growth

After treatment, the cells are cultured for an expression period of 48 h for L5178Y or 72 h for TK6 for DNA damage processing and mutation fixation. Cell densities are measured approximately 24 h following treatment and adjusted to 2×10^5 cells/mL with fresh F_{10P}. On completion of the 48- or 72-h expression period, cell densities are measured again. The cell densities from each expression day are used in calculating the relative suspension growth (RSG) and the relative total growth (RTG) for L5178Y or the RS for TK6 [see (OECD 490, 2016)]. Cultures with cell densities less than 2×10^5/ml are not used for cloning and mutagenicity measurements.

6.3 Cloning (Mutant Selection)

Trifluorothymidine (TFT) stock solution is made with 10 mg TFT in 100 ml physiological saline and stored in a foil-wrapped bottle. The stock solution is filter sterilized and can be dispensed in 15-ml aliquots in sterile tubes and stored at −20°C for up to 3 months.

For cloning, each culture is centrifuged, and the cell pellet resuspended in F_{20P} (cell culture medium with 20% serum) at a density of 2×10^5 cells/mL. The cells should be single cell suspensions, so that individual cells are plated and the colonies that form are from single cells. The cultures are incubated for 30 min to minimize trauma and allow them to adapt to the medium. The cells are then diluted to the appropriate densities to plate for TFT resistance and cell viability.

For the TFT resistant plating (mutant colonies), the cell concentrations are adjusted to 1×10^4/ml in F_{20P} for L5178Y and 2×10^5/ml for TK6 cells. Then TFT (3 mg/ml) is added to the selection flask. Using a multichannel pipette, 200 ml of each TFT containing suspension is placed into each well of 4 flat-bottomed 96-well plates. For L5178 the final density is 2000 cells/well; for TK6 it is 40,000 cells/well. Colonies are identified by low power microscope or by visual observation. Small colonies are defined as less than a quarter of the diameter of the well while large colonies are more than a quarter of the diameter of the well. The morphology is generally compact for small colonies and may be diffuse for large colonies.

For the determination of plating efficiency, the cultures are adjusted to 8 cells/mL in media without TFT and 200 μL per well are aliquoted into two 96-well flat-bottom microtiter plates (~1 or 2 cells/well) for the counting of survivors. The microtiter plates are incubated at 37°C in a humidified incubator with 5% CO2-in-air for 11–14 days for the L5178Y *Tk* mutants. An additional period of 10–14 days is required for the TK6 slower growing colonies to appear.

7 DATA ANALYSIS

7.1 Mutant Frequency (Mutant/Survivor)

The mutant frequency (MF) is determined by the plating efficiencies of mutant colonies (PE_M) and adjusted with plating efficiencies of viable cells (PE_V) from the same culture. See OECD 490 for details (OECD 490, 2016).

7.2 Assay Acceptibility

- The positive control must demonstrate the assay is properly conducted and that small or late growing colony mutants were detected. This is demonstrated by a significantly

induced small colony mutant (MLA) or late growing colony (TK6) mutation frequency in the positive controls.
- RS/RTG >10%
- Spontaneous mutation frequency is 50–170 × 10^{-6} for MLA and ~3.1 ± 1.1 × 10^{-6} for TK6
- Cloning Efficiency for negative control: 65–120% for MLA and >65% for TK6
- Suspension Growth for negative control (corrected for cytotoxicity during treatment and during expression): 8- to 32-fold for the MLA

7.3 Criteria for Positive and Negative Results

These criteria are found in OECD 490 and represent the work of several IWGT MLA Workgroups.

7.3.1 For MLA

- A positive test chemical response requires an induced MF of at least 126 × 10^{-6} for the microwell version of the assay. This is termed the global evaluation factor (GEF). A compound is called negative if it does not meet the criteria for a positive response when the RTG reaches 10–20%.

7.3.2 For TK6

- At least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control.
- There is a dose-related trend
- Any of the results are outside the distribution of the historical negative control.

8 SUMMARY

Mammalian TK mutagenicity assays are recommended for assessment of genetic interactions leading to mutations reflecting heritable sequence changes in the DNA. Two systems described here have been used in laboratories around the world for genotoxicity assessment of chemicals and agents. This paper provides a detailed protocol for use of one or the other of the assays in assessment of NM genotoxicity. NM are a diverse set of agents, often with unique chemical and physical properties that can impact the assays in ways both expected and unexpected. NM generally require special handling for valid assessment of NM, with attention to properties that affect their assessment, such as agglomeration and distribution within biological systems, as well as properties that impact the assays themselves. A detailed protocol is provided for assessment of NM mutagenicity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization: RE; Writing—Original Draft Preparation: TC; Writing—Review and Editing: RE, TC, MD; Methodology: TC, MD. The graphical abstract was created by MD.

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Pre-validation of a reporter gene assay for oxidative stress for the rapid screening of nanobiomaterials

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Engineered nanomaterials have been found to induce oxidative stress. Cellular oxidative stress, in turn, can result in the induction of antioxidant and detoxification enzymes which are controlled by the nuclear erythroid 2-related factor 2 (NRF2) transcription factor. Here, we present the results of a pre-validation study which was conducted within the frame of BIORIMA ("biomaterial risk management") an EU-funded research and innovation project. For this we used an NRF2 specific chemically activated luciferase expression reporter gene assay derived from the human U2OS osteosarcoma cell line to screen for the induction of the NRF2 mediated gene expression following exposure to biomedically relevant nanobiomaterials. Specifically, we investigated Fe_3O_4-PEG-PLGA nanomaterials while Ag and TiO_2 "benchmark" nanomaterials from the Joint Research Center were used as reference materials. The viability of the cells was determined by using the Alamar blue assay. We performed an interlaboratory study involving seven different laboratories to assess the applicability of the NRF2 reporter gene assay for the screening of nanobiomaterials. The latter work was preceded by online tutorials to ensure that the procedures were harmonized across the different participating laboratories. Fe_3O_4-PEG-PLGA nanomaterials were found to induce very limited NRF2 mediated gene

expression, whereas exposure to Ag nanomaterials induced NRF2 mediated gene expression. TiO_2 nanomaterials did not induce NRF2 mediated gene expression. The variability in the results obtained by the participating laboratories was small with mean intra-laboratory standard deviation of 0.16 and mean inter laboratory standard deviation of 0.28 across all NRF2 reporter gene assay results. We conclude that the NRF2 reporter gene assay is a suitable assay for the screening of nanobiomaterial-induced oxidative stress responses.

KEYWORDS

Nrf2, nanomaterial, interlaboratory validation, oxidative stress, nanotoxicology

Introduction

It is a well-established paradigm that ambient particulate matter as well as engineered nanomaterials can trigger oxidative stress (Li et al., 2008; Stone et al., 2017). Under normal physiological conditions, reactive oxygens species (ROS) are continuously formed and immediately neutralized by antioxidant defences such as glutathione (GSH) and an array of antioxidant enzymes. However, under conditions of excessive ROS production, which may occur in cells exposed to engineered nanomaterials or other toxicants, the natural antioxidant defences of the cell may be overwhelmed (Sies and Jones, 2020). Oxidative stress is characterized by a cellular depletion of GSH while oxidized glutathione (GSSG) accumulates. Cells respond to this drop in the GSH/GSSG ratio by several protective or damage related signalling responses (Aguilano et al., 2014).

The NRF2–KEAP1 system plays a key role in maintaining redox homeostasis in eukaryotes (Sies and Jones, 2020). KEAP1 (Kelch-like ECH-associated protein 1) acts as a cysteine thiol-rich sensor of redox insults, whereas NRF2 (nuclear erythroid 2-related factor 2) is a transcription factor that regulates electrophile responsive element (EpRE)-mediated gene expression to switch on a battery of cytoprotective genes. Upon associating with other transcription factors, NRF2 binds to the EpRE and activates EpRE-mediated gene expression, including the genes encoding for detoxifying enzymes and proteins, such as glutathione peroxidase (GPx), NAD(P)H-quinone oxidoreductase (NQO1), superoxide dismutase (SOD), catalase (CAT), peroxiredoxin (PRx), glutathione S-transferase (GST), γ-glutamylcysteine synthetase (γ-GCS) and glutamate-cysteine ligase (GCL) genes. At higher levels of oxidative stress, this protective response is overtaken by cytotoxicity (Li et al., 2008).

Several recent *in vitro* studies reported the activation of the NRF2 pathway following exposure to a variety of engineered nanomaterials, including CeO_2 nanomaterials (Choi et al., 2021), SiO_2 nanomaterials (Cui et al., 2021), and ZnO nanomaterials (Zhang et al., 2021). Moreover, Kim et al. (2021) investigated seven different metal oxides (CuO, Co_3O_4, NiO, TiO_2, CeO_2, Fe_2O_3, and ZnO) using the ARE-NRF2 Luciferase KeratinoSens™ assay that is based on stably transfected immortalised human keratinocytes (HaCaT). CuO nanomaterials but not Co_3O_4, NiO, TiO_2, CeO_2, Fe_2O_3, or ZnO nanomaterials induced a positive response. The latter assay is recognized as a Test Guideline by the OECD since 2018 (Test No. 442D: *In vitro* Skin Sensitisation). Using a NRF2/ARE Responsive Luciferase Reporter HEK293 Cell Line, it has been shown that CuO, Mn_2O_3 and ZnO nanomaterials strongly induce the NRF2 mediated gene expression, while a recent study showed that Fe_2O_3 materials of different sizes induced limited gene expression in these reporter cells (Seleci et al., 2022). Using HEK293 cells, Ag nanomaterials have also been shown in several studies to trigger an NRF2 response in a range of different cell types (Miranda et al., 2022, and see other references therein).

Nanotoxicological studies have been conducted using a plethora of cell-based assays but there is a need for robust (validated) assays that are suitable for high-throughput screening of nanomaterials to improve safety assessment practices (Nymark et al., 2020). In the current study, we applied a reporter gene assay for the screening of oxidative stress induction by nanobiomaterials. This pre-validation study was performed within the EU-funded research project BIORIMA ("biomaterial risk management"). The overarching goal of the BIORIMA project has been to develop a risk management framework for nanobiomaterials used in medical devices and advanced therapy medicinal products (Giubilato et al., 2020). Hazard assessment of nanobiomaterials is one of the important elements of this framework and the approaches to assess the hazard potential of nanobiomaterials can either be based on methods adopted from classical toxicology (of chemicals and other particles) or on alternative methods, including *in vitro* and *in vivo* methods and *in silico* modelling (Giubilato et al., 2020).

In the present study, we pre-validated a reporter gene assay which is based on human osteoblastic osteosarcoma U2OS cells that express luciferase through transfection with a vector carrying antioxidant response elements (ARE) upstream of a luciferase reporter gene (van der Linden et al., 2014). Participating laboratories were recruited from the BIORIMA consortium, of

which 7 laboratories fully completed all experiments. Cells were exposed to Fe_3O_4-PEG-PLGA (Fe_3O_4 -PolyEthylene Glycol - PolyLactide-co-Glycolide Acid) nanomaterials and to Ag and TiO_2 "benchmark" nanomaterials. The Fe_3O_4-PEG-PLGA nanomaterials are envisioned for a variety of applications in medicine, including as a magnetic hyperthermia agent, an *in vivo* imaging/contrast agent, and an active targeting and drug delivery agent (Cazzagon et al., 2022), and for this reason, these materials were selected as one example of a relevant nanobiomaterial.

Materials and methods

Reagents

Curcumin (Sigma cat no. C1386), dichlorvos (Sigma cat no. 45441), and mannitol (Sigma cat no. M9647) were purchased from Sigma Aldrich (Amsterdam, Netherlands), and dimethyl sulfoxide (DMSO) (Arcos cat no. 167852500) was purchased from Acros Organics (Geel, Belgium). Dulbecco's Modified Eagle Medium with Ham's Nutrient Mixture F-12 (1:1) (DMEM/F12) without phenol red (Gibco cat no. 31331-028), Trypsin 0.5% EDTA (10x) (Gibco cat no. 15400-054), nonessential amino acids (NEAA) (Gibco cat no. 11140-035), and phosphate-buffered saline (Gibco cat no. 20012019) were from Gibco (Carlsbad, CA), geneticin (G418) (Duchefa cat no. G0175001) from Duchefa (Haarlem, Netherlands), and penicillin/streptomycin, pH 7.4 (P/S) (Invitrogen cat no. 15070063) from Invitrogen (Breda, Netherlands). Fetal bovine serum (FBS) (Gibco cat no. 10270-106) and dextran-coated charcoal-stripped fetal calf serum (DCC-FCS) (Gibco cat no. 12676029) were both purchased from Gibco.

Nanobiomaterials

Fe_3O_4-PEG-PLGA nanomaterials were provided by Colorobbia Holding S.p.A (Firenze, Italy) in the framework of the BIORIMA research project and synthesized as described (D'Elios et al., 2018). Both Ag and TiO_2 nanomaterials (designated NM300K and NM101, respectively) were from the nanomaterial repository of the Joint Research Center of the European Commission (Ispra, Italy). Ag nanomaterials were provided as a suspension. The NANOGENOTOX protocol was used for dispersion of TiO_2 (Farcal et al., 2015).

Characterization of particles

Small angle X-ray scattering

The dissolution and aggregation of the Ag nanomaterials was monitored by small angle X-ray scattering (SAXS) following incubation for 18 days at 37°C in MEM media (Invitrogen cat no. 51200) supplemented with 4% FBS (Sigma cat no. F7524), 1% Glutamax (Invitrogen cat. no. 35050-038), 1% non-essential amino acids (Invitrogen cat no. 11140), 1% sodium pyruvate (Sigma cat no. S8636), 1% penicillin-streptomycin (Invitrogen cat no. 15140-122) and 1% HEPES (Invitrogen cat no. 15630). SAXS measurements were carried out on Xeuss 2.0 (Xenocs) and ChemSaxs (lab design, CEA) high-resolution X-ray spectrometers in Kapton capillaries at a concentration of 0.5 mg/ml. The signal of the baselines was subtracted and data were fitted with PySAXS software (https://pypi.org/project/pySAXS/). SAXS experiments were performed by one of the participating laboratories.

Transmission electron microscopy

TEM was performed by using a FEI TECNAI F20 microscope operating at 200 keV. The suspension was drop-casted on a holey carbon film supported by a gold grid. The specimen was then dried at 60°C. To gather information about particles morphology the images were taken in phase contrast mode and high-angle annular dark-field scanning transmission mode (HAADF-STEM). High resolution (HREM) and Selected Area Electron Diffraction (SAED) analyses were performed to investigate the crystalline phase structure and composition. To calculate the mean particle diameter more than 100 particles were measured. TEM experiments were performed by one of the participating laboratories.

Dynamic light scattering

Hydrodynamic sizes and zeta potential of Fe_3O_4-PEG-PLGA nanomaterials were determined as previously described in the NanoREG project (Bhattacharya et al., 2017). In short, concentrations of the test samples were adjusted from the 1 mg/ml respective stock suspensions using either endotoxin free water or the medium with or without FBS to a concentration of 25 μg/ml for the measurements. Particle size distribution and zeta potential of the test samples were measured by dynamic light scattering (DLS) technique using Malvern Zetasizer Nano ZS. Three measurements with no pause were taken for particle size distribution and for the zeta potential values of each test material at 0, and 24 h at a temperature of 25°C. DLS experiments were performed by one of the participating laboratories.

Endotoxin detection

The Limulus Amoebocyte Lysate (LAL) assay was applied to detect bacterial endotoxin contamination as described earlier (Kroll et al., 2013; Eder et al., 2022). The Limulus Amoebocyte Lysate PYROTELL®–T assay was purchased from Associates of Cape Cod, Inc. (East Falmouth, MA) and used according to the manufacturer's instructions. Data analysis was performed using PYROS® Software (Associates of Cape Cod, Inc.).

Cell culture

U2OS-NRF2 cells were kindly provided by Bio Detection Systems (Amsterdam, Netherlands). The human osteoblastic osteosarcoma U2OS-NRF2 cells (van der Linden et al., 2014) express two oligos containing four different EPRE sequences: 1 × consensus EPRE (TCACAGTGACTAAGCAAAAT), 1 × hNQO1 EPRE (TCACAGTGAC TCAGCA-GAAT), 1 × hGCLM EPRE (AGACAATGACTAAGCAGAAA) and 1 × hGCLC EPRE(TCACAGTCAGTAAGTGATGG). The two oligos were ligated into a promoter-less luciferase reporter-construct pLuc. Because the U2OS cells express the NRF2 pathway endogenously, a selection construct (pSG5-neo) was used. The cells were cultured in DMEM/F12 supplemented with 10% FCS and penicillin/streptomycin (final concentrations 10 U/ml and 10 µg/ml, respectively) (designated as growth medium). Once per week, 200 µg/ml G418 was added to the culture medium to maintain selection pressure. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

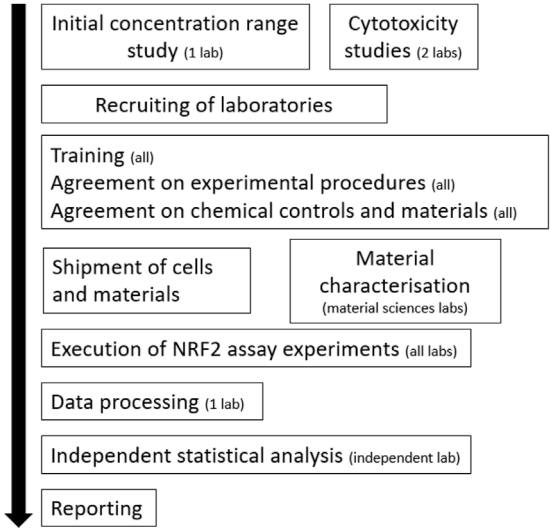

FIGURE 1
Workflow of the pre-validation experiments. The number of laboratories involved is indicated.

Cell viability assay

Cytotoxicity of nanobiomaterials was evaluated by the Alamar blue (resazurin) assay as described (Keshavan et al., 2021). The cell viability experiments were performed by one of the participating laboratories, prior the "round robin" pre-validation experiments. The cells were trypsinized, counted, and resuspended in cell culture medium without phenol red and supplemented with 5% dextran-coated charcoal-stripped FCS (DCC–FCS), to a final concentration of 10^4cells/well (100 µl). Cells were seeded in 96-well plates and exposed to test materials or were maintained in DCC–FCS alone (negative control). The assay reagent (Thermo Scientific, Sweden) (10% [v/v] solution of AlamarBlue® reagent) was added to each well to monitor the cellular metabolic function. The samples were analyzed using a spectrophotometer (Tecan Infinite® F200).

Reporter gene assay

The potential induction of NRF2 mediated gene expression by nanobiomaterials was tested by measuring the induction of luciferase activity in the NRF2-U2OS cells. Protocols are available upon request. In brief, the cells were trypsinized, counted, and resuspended in cell culture medium without phenol red and supplemented with 5% dextran-coated charcoal-stripped FCS (DCC–FCS) at a final concentration of 10^4cells/well (100 µl) in a 96-well plate without using the most outer wells. The plates were incubated for 24 h in a humidified atmosphere at 37°C under 5% CO_2. Following this pre-incubation one reference plate was exposed containing 9 serial dilutions in the range of 1×10^{-4} M to 1×10^{-8} M (log10 dilution steps) of the reference compound curcumin, as well as a positive control dichlorvos (1×10^{-5}–7×10^{-7} M) and a negative control mannitol (1×10^{-3}–1×10^{-5} M). Dichlorvos was included as a positive control as it is known to induce a response in this assay, while the negative control (i.e., mannitol) should not. Curcumin was chosen as reference compound, as it usually results in a dose-effect response in the current assay. It is good practice to select different chemicals as reference chemical and positive control. The cells were exposed to reference compounds by adding the compounds from a 200 x concentrated stock solution in DMSO to exposure medium (5% DCC-FCS in DMEM/F12 without phenol red). Following exposure to the test materials, cells were further processed for the luciferase induction assay. Cells were rinsed using PBS followed by lysis through 30 µl low salt buffer (Tris, 25 mM, DTT 2.0 mM, CDTA 2.0 mM), and a subsequent freezing step at −80°C ensured complete cell lysis. Luciferase was measured using a flash mix protocol (BDS, Amsterdam, Netherlands). The flash mix or illuminate mix contained 20 mM tricine, 1.07 mM $(MgCO_3)4\ Mg(OH)2.5\ H_2O$, 2.67 mM $MgSO_4$ x 7 H_2O, EDTA 0.1 mM, DTT 1.5 mM, D-Luciferine 539 mM, ATP 5.49 mM. The measurements were performed in the different laboratories using a luminometer with two injectors, one to initiate the reaction (through the addition of the Luciferin present in the illuminate mix) and one for stopping the enzymatic reaction with NaOH. The reaction was thus stopped by adding 100 µL of 0.2 M NaOH. A threshold of induction factor of 1.5 was set for the NRF2 mediated gene expression, as described before (van der Linden et al., 2014).

Design of "round robin" pre-validation

Fe_3O_4-PEG-PLGA nanomaterials were selected as a representative and novel nanobiomaterial for the present study. These nanomaterials are envisioned both for therapeutic and diagnostic applications. The "benchmark" TiO_2 nanomaterials were included as an inert (non-cytotoxic) nanomaterial and Ag nanomaterials were included as a nanomaterial that most likely would elicit NRF2 mediated gene expression (based on the available literature, see above), though cytotoxicity at higher concentrations of the latter nanomaterials could not be excluded. Additional positive and negative chemical controls (dichlorvos and mannitol) were included for the assay based on the manufacturer's recommendations.

The participating laboratories were trained (online) on the execution of the NRF2 reporter gene assay, quality control measures, and data analysis (for a schematic of the workflow, refer to Figure 1). The following laboratories/institutions participated in the pre-validation study: Karolinska Institutet, Wageningen University, University of Torino, Université Grenoble-Alpes, Edinburgh Napier University, University of Rome Tor Vergata, Université Paris Cité, and Tokyo University of Science. However, one of these laboratories only tested Fe_3O_4-PEG-PLGA and not the other "benchmark" nanomaterials and the results are therefore shown separately. Protocols were extensively discussed and agreed upon during online meetings and tutorials. Chemicals and cell culture reagents were procured from the same source, and the NRF2-U2OS cell line was distributed to all the laboratories. The plate layout for the reporter gene assay was decided. Each experiment thus included one reference plate and three experimental plates. The three upper rows (B-C-D) of the reference plate as well as each experimental plate contained a full concentration range of the reference compound curcumin dissolved in DMSO. The lower part (rows E-F-G) contained the positive and negative control (reference plate) or one of the three nanobiomaterials under investigation (Fe_3O_4-PEG-PLGA, TiO_2, Ag). The participating laboratories also harmonized the exposure conditions. Hence, Fe_3O_4-PEG-PLGA and Ag nanomaterials were diluted from a stock of 3000 µg/ml in dispersant provided with the particles at a concentration range of 0.21 µg/ml–3000 µg/ml followed by a second 30 x dilution step in exposure medium to an exposure range of 0.001–100 µg/ml. For TiO_2, freshly prepared suspensions were made using the NANOGENOTOX dispersion protocol (Farcal et al., 2015). The reporter cells were exposed for 24 h in a humidified atmosphere at 37°C under 5% CO_2.

Data analysis

Data were exported to Excel (Microsoft) for further processing. Cytotoxicity was expressed as % viability towards the unexposed cells. For the NRF2 reporter gene experiments, the results were presented as the Induction Factor (IF), which is the measured relative light unit (RLU) value divided by the mean RLU value of the solvent control. When the induction factor of curcumin was over 8, the NRF2-U2OS reporter gene assay was regarded to be effective. Samples presenting 1.5 fold or higher induction were considered as inducers of NRF2 mediated-gene expression (van der Linden et al., 2014). Graphs were prepared in Prism 9.0 (GraphPad Software, Inc.) by analysing data using non-linear curve fitting (agonist versus response). To evaluate the variability of results and reproducibility of the assay, both the intra-laboratory and inter laboratory standard deviations were calculated across all NRF2 reporter gene assay results and plotted in a heatmap. Statistical analysis was performed using GraphPad Prism version 8.3.0.

Interlaboratory standard deviation of the assay results of all participating laboratories was calculated in accordance with ISO standards 5725-1 and 5725-2 for accuracy (trueness and precision) of measurement methods and results.

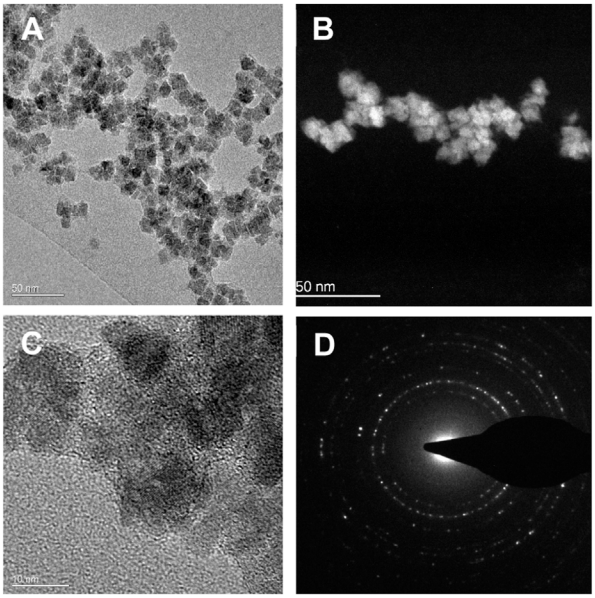

FIGURE 2
Transmission electron microscopy images of as-synthesized Fe_3O_4-PEG-PLGA nanomaterials. **(A)** TEM phase contrast image; **(B)** HAADF-STEM image; **(C)** HREM phase contrast image; **(D)** SAED polycrystalline pattern rings. Scale bars: **(A,B)** 50 nm; **(C)** 10 nm.

Results

Characterisation of nanobiomaterials

The Fe_3O_4-PEG-PLGA nanomaterials obtained from Colorobbia and the corresponding dispersant were evaluated for sterility (endotoxin content). Both were found to contain

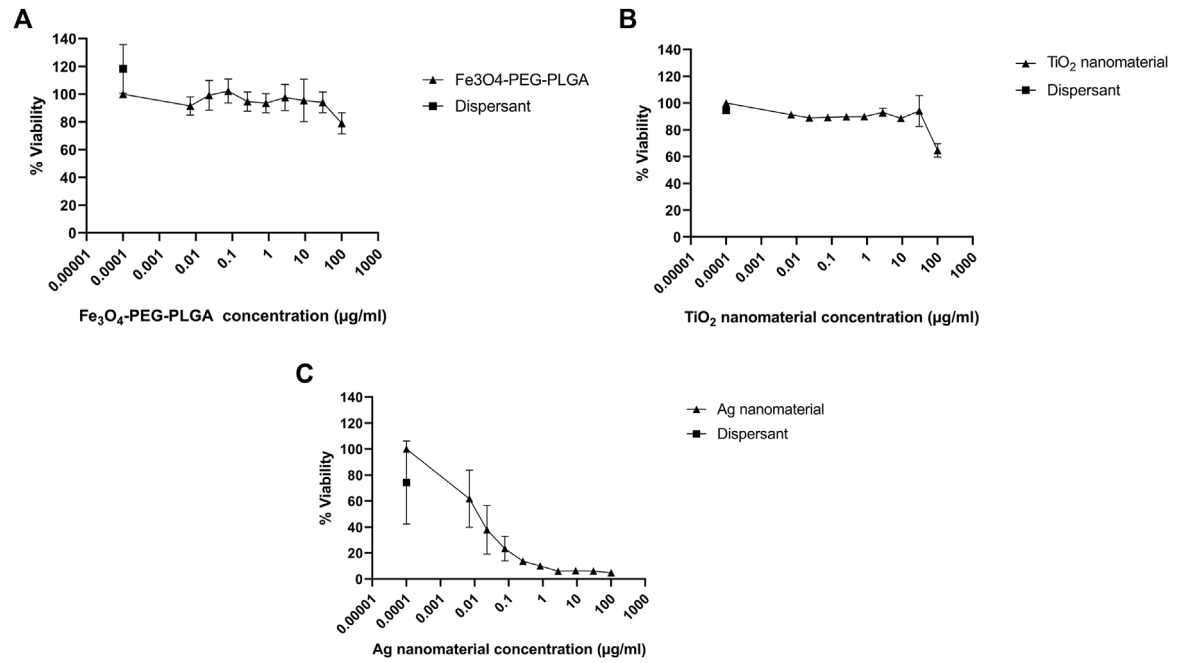

FIGURE 3
Cytotoxicity assessment. U2OS cells were exposed for 24 h to **(A)** Fe_3O_4-PEG-PLGA, **(B)** TiO_2 nanomaterials, and **(C)** Ag nanomaterials or dispersants and cell viability (metabolic capacity) was evaluated using the Alamar blue assay. Data are mean values ± S.D. of three independent experiments.

endotoxin levels below the US FDA-mandated level for medical devices (data not shown). The Fe_3O_4-PEG-PLGA nanomaterials were visualized by TEM. TEM phase contrast images (Figure 2A) and HAADF-STEM images (Figure 2B) indicated regular morphology with a mean particle diameter of 12 ± 4 nm. The higher magnification HREM phase contrast images (Figure 2C) disclosed a cubic crystal structure consistent with the magnetite lattice, and polycrystalline pattern rings collected by SAED (Figure 2D) were indexed as crystalline magnetite, identified as the unique phase composition. The benchmark materials were fully characterised, see Comero et al. (2011) for Ag, and Rasmussen et al. (2014) for the TiO_2 nanomaterials.

SAXS analysis showed that there was little or no dissolution of the Ag nanomaterials following incubation at 37°C for 18 days in culture media. The average size of these nanomaterials did not change during incubation (15 ± 0.2 nm and 15 ± 0.2 nm at t = 0 and t = 18 days, respectively). The nanobiomaterials were also analysed with respect to hydrodynamic diameter and zeta potential in the relevant cell culture medium. Previously the dissolution of the Fe_3O_4-PEG-PLGA nanomaterials in cell culture media was shown to be less than 0.5% within 24 h (data not shown). Together the data indicated that all the test materials were stable following incubation in cell culture medium for 24 h at the exposure conditions for the NRF2 reporter gene assay (Supplementary Figures S1A,B).

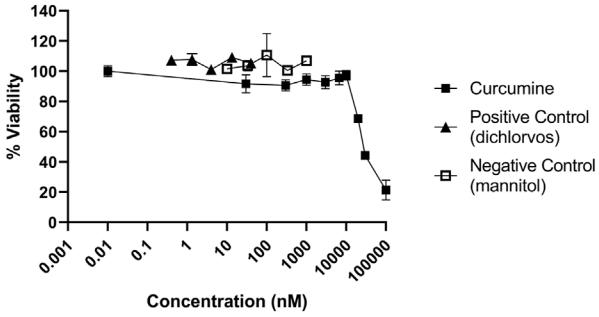

FIGURE 4
Cytotoxicity of control chemicals used in the NRF2 reporter gene assay. U2OS cells were exposed to curcumin, dichlorvos, and mannitol for 24 h and evaluated using the Alamar blue assay.

Cytotoxicity assessment

For a correct interpretation of the results from the reporter gene assay, the potential of the test materials to reduce cell viability should be assessed. To this end, the Alamar blue assay was used. Fe_3O_4-PEG-PLGA nanomaterials were non-cytotoxic towards U2OS cells and only a slight decrease in cell

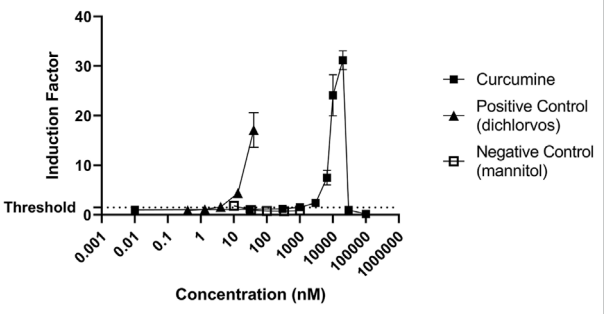

FIGURE 5
Induction of NRF2 mediated gene expression by the reference compound (curcumin) and negative (mannitol) and positive controls (dichlorvos). The results are presented as induction factor, the fold induction over the solvent control. The data are presented as mean values ± S.D. of three independent experiments.

viability (metabolic capacity) was evidenced at the highest tested concentration of 100 µg/ml (Figure 3A). Similarly, TiO_2 nanomaterials were non-cytotoxic at low concentrations but a markedly decreased viability at the highest concentration of 100 µg/ml (Figure 3B) was noted. In contrast, for Ag nanomaterials, a dose-dependent loss of cell viability was observed (Figure 3C). The potential cytotoxic effects of the reference compounds curcumin, dichlorvos (positive control) and mannitol (negative control) were also evaluated (Figure 4). Neither dichlorvos or mannitol affected cell viability of the U2OS cells, while curcumin at a concentration of 500 nM and higher reduced U2OS cell viability in a dose-dependent manner (Figure 4).

Nuclear erythroid 2-related factor 2-reporter gene assay

Next, the induction of the NRF2 pathway was assessed. NRF2-U2OS cells were exposed to increasing concentrations of the reference compound (curcumin), and to the positive and negative controls (Figure 5). Both the reference compound and the positive control (dichlorvos) induced NRF2 mediated gene expression while exposure to mannitol did not (Figure 5).

Eight laboratories participated in the "round robin" pre-validation study, of which seven used Fe_3O_4-PEG-PLGA, Ag, and TiO_2 nanomaterials (Figure 6), whereas one partner only used Fe_3O_4-PEG-PLGA nanomaterials (Supplementary Figure S2). The results consistently showed that TiO_2 did not induce NRF2-mediated gene expression. However, exposure to Ag nanomaterials induced NRF2-mediated gene expression in a dose-dependent manner in all experiments (Figures 6A–G). Some differences could be observed in the magnitude of responses (i.e., induction factor) of similar concentration in the different laboratories. It was consistently found that the NRF2 mediated gene expression declined at the highest concentrations which likely is due to the cytotoxicity following exposure to the Ag nanomaterials at the higher concentrations. Finally, following exposure to Fe_3O_4-PEG-PLGA minimal induction of NRF2-mediated gene expression was observed (Figures 6A–G). Hence, while three of the participating laboratories reported no induction, the results from 5 other laboratories showed a minor induction at 30 or 100 µg/ml, while some reported a lower induction factor for the 100 µg/ml samples compared to 30 µg/ml. Finally the inter and intra-laboratory standard deviations of the assay results were calculated.

The inter-laboratory standard deviation ranged from 0.044 to 1.221 with a mean of 0.28 (Figure 7). The mean intra-laboratory standard deviation was 0.16 (Supplementary Figure S3).

To verify the lack of interference of the test materials with the measurement of luciferase activity, the U2OS-NRF2 cells were fixed at the end of exposure by adding 50 µl of paraformaldehyde at 4% in PBS for 30 min at room temperature just before cell lysis to perform the luciferase induction assay as described above. No interference was observed (data not shown).

Discussion

In this study, we aimed to perform a pre-validation study of a NRF2 reporter gene assay to screen for activation of NRF2 mediated gene expression following exposure to nanobiomaterials, as a proxy for oxidative stress. The pre-validation was conducted through the participation of eight laboratories. Fe_3O_4-PEG-PLGA nanomaterials were selected as a representative nanobiomaterial and results were compared to "benchmark" nanomaterials from the JRC namely TiO_2 (NM101) and Ag (NM300K) along with positive and negative chemical controls. For the TiO_2 nanomaterials, none of the participating laboratories observed an induction above the threshold. For the Fe_3O_4-PEG-PLGA nanomaterials, some laboratories measured an induction just above the threshold while for others the induction levels did not reach the threshold. All laboratories could detect a dose dependent induction following exposure to the Ag nanomaterials (though with different induction factors) indicating that the NRF2 reporter gene assay can be easily applied by different laboratories. Overall, interlaboratory standard deviation was acceptable and the NRF2 reporter gene assay for quantifying oxidative stress caused by nanomaterials is suitable for application in different laboratories. Based on these preliminary findings, we suggest that the assay may be considered for formal validation as an assay for rapid screening of nanobiomaterials.

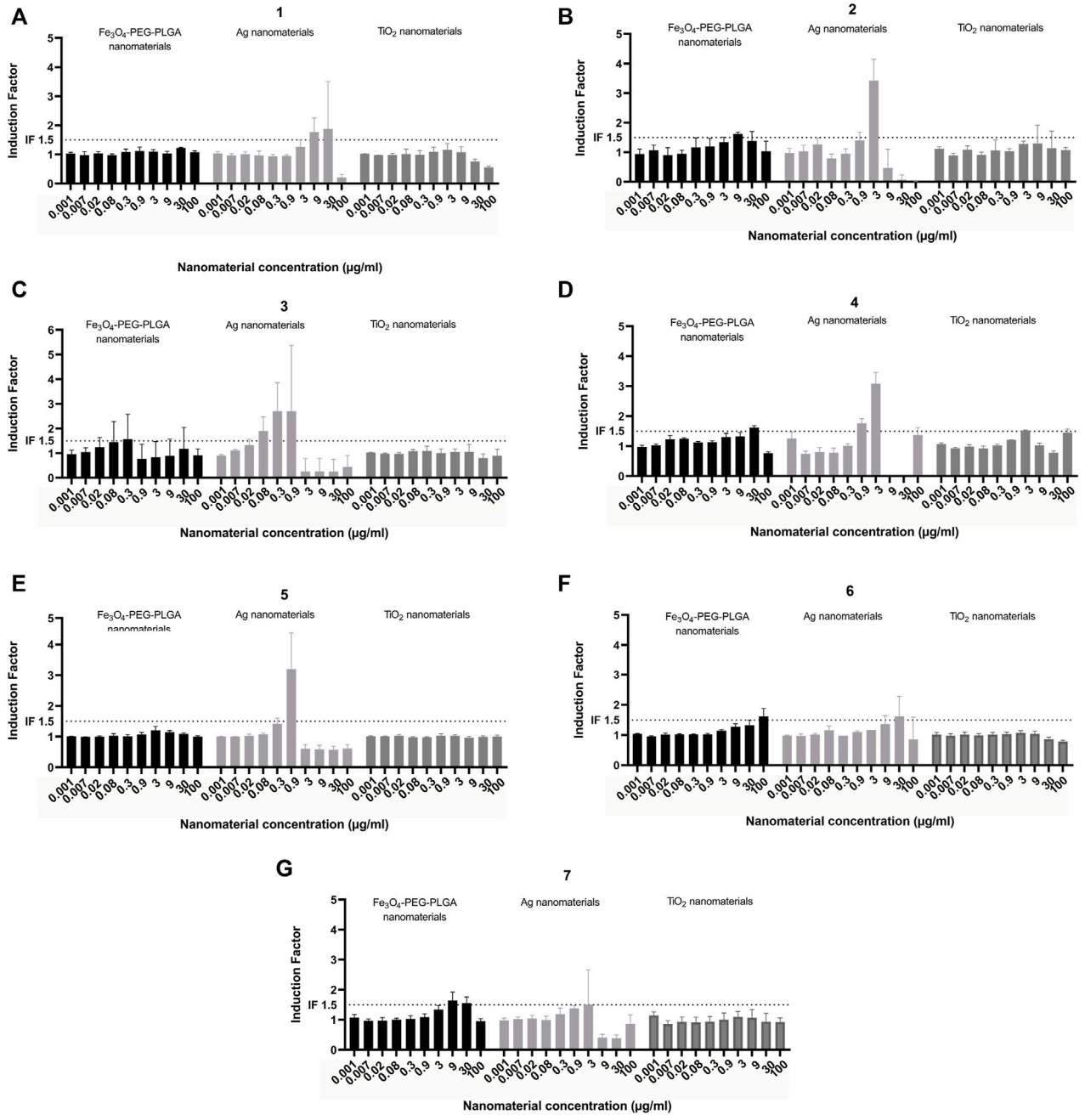

FIGURE 6
Interlaboratory study. Induction of NRF2 mediated gene expression following exposure of Fe_3O_4-PEG-PLGA, Ag nanomaterials and TiO_2 nanomaterials. Each graph **(A–G)** represents the results of an individual laboratory. Each experiment was performed according to the same harmonized protocol. The results are presented as induction factor, the fold induction over the solvent control. The data are presented as mean values ± S.D. of three independent experiment. The numbers represent the individual participating laboratories. Black bars: Fe_3O_4-PEG-PLGA, light grey bars: Ag; dark grey bars: TiO_2.

Engineered nanomaterials can induce ROS production *via* several mechanisms such as the Fenton reaction, redox cycling, and radical generation (Bi and Westerhoff, 2019), which in turn can activate the NRF2 mediated gene expression. Several previous studies have shown that metal oxides including CuO and ZnO nanomaterials can elicit NRF2 activation (Kim et al., 2021; Zhang et al., 2021). Moreover, Ag nanomaterials exposure was found to trigger NRF2 activation which is thus in line with

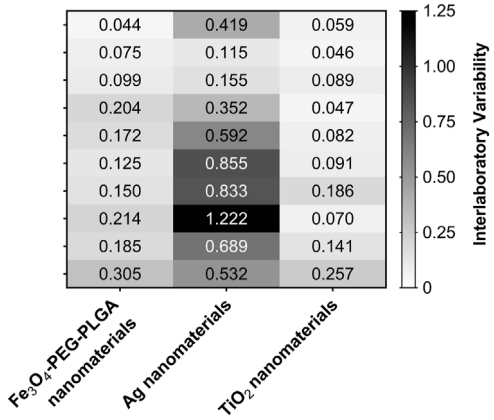

FIGURE 7
Heatmap of the interlaboratory standard deviation of the NRF2 induction results from all participating laboratories. The combinations of concentration and nanomaterials that resulted in higher variability of assay results across the partner laboratories are indicated by darker boxes in the heatmap.

the present findings. The results from the present study showed that Fe_3O_4-PEG-PLGA nanomaterials elicited a very modest activation in U2OS-NRF2 cells. However, with respect to Fe_3O_4-PEG-PLGA, no literature was found on their potential for the induction of NRF2 mediated gene expression. In a recent study different acellular assays along with a HEK293 cell-based NRF2 reporter assay were compared to study the generation of ROS and antioxidant responses of engineered nanomaterials. It is notable that the HEK293 cell-based NRF2 reporter assay did not show any concentration-dependent reactivity for the Fe-based nanomaterials (Seleci et al., 2022), while Fe_3O_4 nanomaterials have been shown to be able to induce oxidative stress in rodents (Wu et al., 2022). The U2OS-NRF2 cells have a low number of receptors expressed, so that the potential for crosstalk between signal transduction pathways is very low. Furthermore, the U2OS cell line has a low overall metabolic capacity so that reactive compounds, or metabolites have a better chance and a higher sensitivity to activate reporter systems. In comparison to the KeratinoSens™ method validated by OECD (Test No. 442D) for *in vitro* skin sensitization which has one ARE-response element upfront of the ARE-reporter construct, the U2OS-NRF2 cells as used in the current study has a tandem of four AREs upstream of the luciferase reporter and may thus be more sensitive to inducers acting *via* the NRF2-pathway. However, care needs to be taken when comparing reporter assays in different cell systems and *in vivo* data as different abundancies of thiol-containing ligands (i.e., GSH and metallothioneins) can influence the presence of intracellular ROS levels (Bobyk et al., 2019) and may thus influence the sensitivity of cell based NRF2 reporter gene assays.

In addition to the aforementioned *in vitro* studies, a number of *in vivo* studies performed in rodents have also shown that nanomaterials can trigger NRF2 activation. For instance, Sun et al. (2012) showed that long-term exposure to TiO_2 nanomaterials induced the expression of NRF2, heme oxygenase 1 (HO-1), and glutamate-cysteine ligase catalytic subunit (GCLC). Other investigators have reported that intratracheal administration of ZnO nanomaterials induced elevation of NRF2 and HO-1 expression in the aorta of mice (Zhang et al., 2021). Furthermore, members of the BIORIMA consortium previously investigated the role of NRF2 in pulmonary inflammation following exposure to ZnO nanomaterials using *Nrf2*-null mice (Sehsah et al., 2019). Mice were administered 20 nm ZnO nanomaterials via pharyngeal aspiration and the study demonstrated infiltration of inflammatory cells in the lung of mice, but minimally induced NRF2-dependent antioxidant enzymes. The authors concluded that NRF2 plays a role in negative regulation on ZnO nanoparticle-induced neutrophil migration (Sehsah et al., 2019).

Several studies have been undertaken in recent years to improve the quality of nanotoxicological investigations including a number of interlaboratory comparisons (aka round robins). For instance, a US consortium funded by the NIEHS conducted cell-based assays on a panel of nanomaterials including several forms of TiO_2 and ZnO nanomaterials as well as multi-walled carbon nanotubes focusing on cell viability and cytokine (IL-1β) production (Xia et al., 2013). The importance of using well-characterized nanomaterials and positive and negative controls was emphasized. Several pan-European projects have also addressed the harmonization of *in vitro* test protocols for the assessment of nanomaterials (e.g., Dusinska et al., 2015; Farcal et al., 2015; Kermanizadeh et al., 2016; Piret et al., 2017). These efforts have put a spotlight on the crucial importance of harmonized test protocols while acknowledging that the path to regulatory-relevant results can be both arduous and long (Teunenbroek et al., 2017).

The OECD Working Party on Manufactured Nanomaterials (WPMN) has reviewed the need for adaptation of the existing OECD Test Guidelines (TGs) and Guidance Documents (GDs) as well as developing new TGs and GDs to address nanomaterials (Rasmussen et al., 2019). Indeed, in the frame of the so-called Malta Initiative, 18 European countries, several Directorates-General of the European Commission, the European Chemicals Agency (ECHA), and other organizations collaborate with the aim of making legislation enforceable, in particular for chemicals (Mech et al., 2022). This European action is currently focused on amending the OECD TGs with respect to nanomaterials to ensure that they are fit-for-purpose. The present reporter gene assay which reflects an important biological endpoint namely oxidative stress is well aligned with these efforts, although further validation is certainly required.

Conclusion

In conclusion, we have successfully performed a pre-validation "round robin" using the NRF2 reporter gene assay using Fe_3O_4-PEG-PLGA vs. TiO_2 (NM101) and Ag (NM300K) nanomaterials. The assay was readily adopted by different laboratories. It is worth noting that other reporter gene assays have previously been subjected to validation and that the estrogen receptor (ER) reporter gene assay and androgen receptor (AR)-reporter gene have recently been included in OECD TG 455 and TG 458, respectively. We suggest that the results of the present interlaboratory study may serve as a starting point for a larger validation study to develop the NRF2 gene reporter assay for the screening of the induction of oxidative stress responses triggered by nanobiomaterials. Indeed, high-throughput screening using *in vitro* assays could speed up the hazard assessment of nano (bio) materials (Fadeel et al., 2018).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

NFR2 pre-validation experiments at the participating laboratories were done by SM, LdH, IM, GA, EB, CR, DB, MC, GH, EM, LY, LC, FC, AP, AB, SB, and GI. Characterization of materials including endotoxin assessment: JS, MB, AC, OT, and SD. LdH and HB analyzed the NRF2 data, and KE and AM performed the statistical analysis. BF and HB conceptualized and designed the study and wrote the manuscript. All authors reviewed the final version of the manuscript and approved its submission to the journal.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2022.974429/full#supplementary-material

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The colony forming efficiency assay for toxicity testing of nanomaterials—Modifications for higher-throughput

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To cope with the high number of nanomaterials manufactured, it is essential to develop high-throughput methods for *in vitro* toxicity screening. At the same time, the issue with interference of the nanomaterial (NM) with the read-out or the reagent of the assay needs to be addressed to avoid biased results. Thus, validated label-free methods are urgently needed for hazard identification of NMs to avoid unintended adverse effects on human health. The colony forming efficiency (CFE) assay is a label- and interference-free method for quantification of cytotoxicity by cell survival and colony forming efficiency by CFE formation. The CFE has shown to be compatible with toxicity testing of NMs. Here we present an optimized protocol for a higher-throughput set up.

KEYWORDS

nanomaterials, cytotoxicity, cell viability, colony formation, CFE, 12-well format, hazard and risk assessment

Introduction

The nanosize that gives rise to the highly advantageous properties of nanomaterials (NMs) designed for various products and purposes could also induce unintended effects on human health. To develop safe NMs, the safer-by-design (SbD) principle should be followed, whereby toxicity testing is performed in parallel with the development of the NMs (Yan et al., 2019; Sánchez Jiménez et al., 2022). Validated test methods for NM hazard identification are urgently needed, as standard toxicity test protocols often need modifications to avoid biased results. An important aspect of this is the potential interference of the NM with the read-out or reagents of the assay applied, due to the high reactivity of the NMs (MacCormack et al., 2021). This can be a challenge in optical detection methods (light absorption, fluorescence), metabolic assays (chemical reaction between the NMs and the assay components) and enzymatic assays (adsorption of assay molecules (e.g. antibodies, enzymes) on the particle surface) (Kroll et al., 2012; Guadagnini et al., 2013; Lee et al., 2022). Thus, label-free *in vitro* test methods are very beneficial to significantly reduce

the likelihood of interaction and biased hazard identification of NMs. Due to the vast number of NM-based products, it is not possible to test all of them by standard assays, thus higher-throughput toxicity tests are needed.

The cytotoxic effects of chemicals, including NMs, can be determined by different endpoints, such as membrane integrity (e.g. trypan blue assay), metabolic activity (e.g. AlamarBlue, MTT, WST-1 assay), relative cell proliferation (e.g. relative cell growth assay) or label-free impedance analysis (e.g. xCELLigence system). Cell viability can also be measured by the ability of cells to survive and form colonies, which is the endpoint of the colony forming efficiency assay (CFE). Being non-colorimetric and non-fluorescent, the CFE assay is especially suitable for assessment of toxicity of NMs to avoid potential interference.

The CFE assay is applicable for most adherent mammalian cells in culture, and stable cell lines are mostly used. Individual cells are exposed, and each surviving cell will divide and form a colony. This allows for the quantification of cell survival/cell death, and also for the detection of cytostatic effects by evaluating the size of the colonies. Reduced colony size will reflect slowed cell proliferation and growth. The test method has similarities with the plating efficiency assay (part of the OECD test guideline 476), however, for the plating efficiency assay exposure is performed on a confluent cell population grown in monolayer.

The CFE assay was optimized and standardized some years ago for NMs testing by the JRC's Nanobiosciences Unit and validated in a interlaboratory comparison study (Ponti et al., 2014), and it has been used with different *in vitro* systems to assess the cytotoxicity of a wide range of NMs e.g., gold NMs (Coradeghini et al., 2013), silver NMs (Locatelli et al., 2012; El Yamani et al., 2017), titanium oxide NMs (de Angelis et al., 2012; Fenoglio et al., 2013; El Yamani et al., 2017; Lee et al., 2022), zinc oxide NMs (de Angelis et al., 2012; El Yamani et al., 2017), silica NMs (Uboldi et al., 2012; Lee et al., 2022), multi-walled carbon nanotubes (Ponti et al., 2010), copper oxide (Lee et al., 2022), graphene (Won et al., 2022), nickel (Latvala et al., 2016), and cerium oxide NMs (El Yamani et al., 2017; Lee et al., 2022).

In this paper we provide the protocol for an optimized and miniaturized version of the CFE assay for higher through put, moving from Petri dishes to 6-well plates and further to 12-well plates. The assay is easy to perform, and time- and cost-efficient, and found to be very suitable for cytotoxicity testing of NMs. As in general for NM testing, specific considerations should be followed. Toxicity of NMs is dependent on physico-chemial properties, such as size, shape and surface coating. Thus, NMs need to be fully characterized, dispersible in culture medium and stability of the NM dispersion needs to be checked and reported (El Yamani et al., 2017; Elespuru et al., 2022).

Materials and equipment

Materials

Cells (adherent cell line), flasks 25 cm^2 or/and 75 cm^2, 12-well (or 6-well) plates, sterile plastic centrifuge tubes, microcentrifuge tubes, serological pipettes, pipettes and tips, cell culture medium (according to cell line) and additives (e.g serum, Penicillin-Streptomycin), trypsin-EDTA, methylene blue (CAS number 122965-43-9), filtration paper, phosphate buffered saline (PBS), CO_2, distilled water, ethanol, Bürker chamber + Cover slips 22 × 22 mm/Cell counter slides, trypan blue stain 0.4%, ink pen or e-count pen.

Equipment needed

Laminar flow hood, light microscope, automated cell counter/Bürker chamber, pipettes, CO_2 incubator, refrigerator, water bath, vortex, autoclave.

Solutions

Preparation of methylene blue (1%): l g of methylene blue is dissolved in 100 ml of MilliQ water. Filter through filtration paper. It is not necessary to sterilize it. The solution can be kept at room temperature.

Methods

The CFE assay is performed on individual mammalian cells plated out in small inoculum (i.e. 25-200 cells per well) on 12- (or 6) well plates at 1–16 h (h), depending upon growth rate of the cells, before treatment. The cells should not divide after seeding before exposure. Then, cells are exposed to the test compound, positive and negative controls and cultured to allow for colony formation, generally for 5–12 days (d), depending on cell type and their doubling time. The colonies are stained and counted manually or by automated scoring. A brief outline of the steps is given in Figure 1, followed by subsections with more detailed description.

Cell lines and preparation of culture

Human or mammalian cells growing attached to the surface with high cloning efficiency, such as V79, A549 or HepG2 cells (El Yamani et al., 2017; Lee et al., 2022), are commonly used with the assay. Any adherent cells growing with high cloning efficiency can be used. Cells are cultivated in complete culture medium and incubated in culture dishes or flasks in a cell

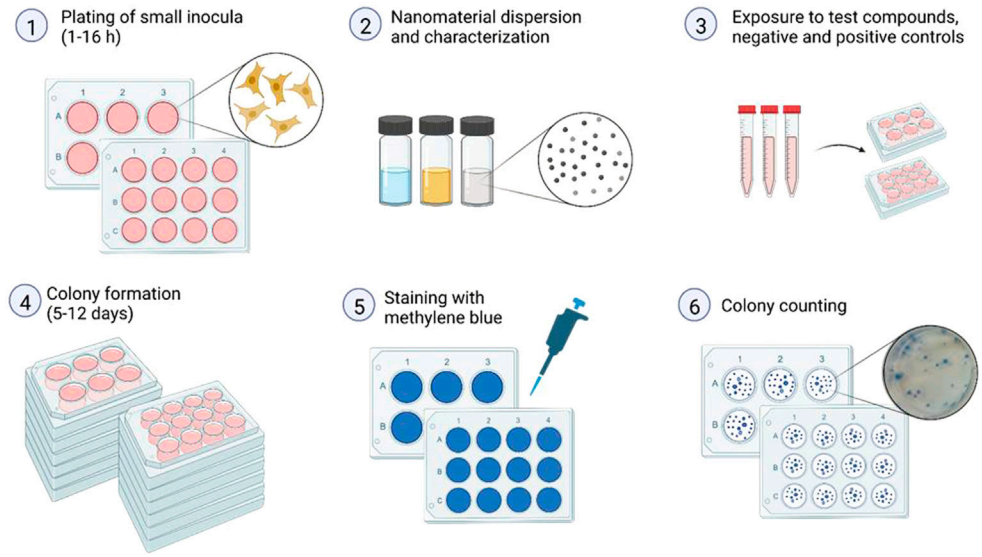

FIGURE 1
Graphical design of the colony forming efficiency (CFE) assay (Created with BioRender.com). 1. Trypsinize and count the cells. Seed the cells in correct density. It is important to mix the suspension prior to plating to ensure an even suspension of cells, as well as to spread the cells evenly in the wells. Remember to label both lid and the plate properly to avoid mix-up. Keep the cells in the incubator. 2. Prepare dispersion of NMs. Perform proper particle characterization. 3. Dilute NMs and controls in culture medium and add to the plates. Remember to make 2 × concentration since there is already half of medium in the well. 4. Leave the plates with the cells in the incubator to form colonies, normally 5–12 days 5. When colonies visible by eye are formed in negative control plates, the colonies should be stained in 1% methylene blue. Add 20 µl of methylene blue into each well and leave for minimum 30 min. Remove the staining solution into waste bottle. To reduce background staining, the plates can be rinsed carefully with water after staining. Leave the plates to dry. 6. Count the colonies. h, hours; d, days.

incubator with humidified atmosphere at 37°C, 5% of CO_2 as described in the standard operating procedure (SOP) for cultivation of the cell line.

Cells are thawed, put into culture medium and cultivated in a cell incubator.

Seeding of cells for exposure

The cells should be sub-cultured at least 2–3 times before being seeded for exposure. Cells should be taken in the exponential growth phase (50–80% confluency) in low passage (max P15). Briefly, seed cells in 12-well plates in low inoculum at 1–16 h before exposure. The time is selected depending on the generation time for the cells, as the cells should not divide between seeding and exposure to be able to expose individual cells.

The number of cells to be seeded per well is dependent on the plating efficiency and proliferation rate of the cell line applied. For human lung epithelial A549 cells, with a rather high plating efficiency and doubling time of about 22 h, it is recommended to seed 30 cells/well in 0.5 ml of cell culture media for 12-well plates, or 50 cells in 1 ml media for 6-well plates. A sequential dilution of the cells to the right concentration is recommended. See the suggested procedure below:

a. Prepare dilution of 1×10^5 cells/ml. Re-suspend well by pipetting and/or vortexing.
b. Prepare further 1×10^4 cells/ml ($10 \times$ dilution of 1×10^5 cells/ml) e.g. 0.1 ml of suspension of 1×10^5 cells/ml plus 0.9 ml of medium. Vortex.
c. Prepare further 1×10^3 cells/ml dilution e.g. 0.1 ml of suspension 1×10^4 cells/ml plus 0.9 ml of medium. Vortex.
d. Prepare dilution of the number of cells you want per ml, e.g. 60 cells/ml (16.7 × dilution of 1×10^3 cells/ml dilution).

Calculate the volume needed for all wells. It is recommended for more robust data to include six replicate exposure wells, three independent experiments. In case of shortage of test substances, the number of replicate wells can be reduced, but this will increase the margin of error. Place the cells in the incubator to settle before exposure to the test substance and controls. Remember to label the plate and the lid properly to avoid mix-up during the experiment.

Preparation of test NM and controls

Prepare vials with 2x final concentrations of the test substance, diluted in cell culture media. Negative control is cells exposed to cell culture media only. A positive control

should always be included to demonstrate responsiveness of the cells. This is especially important when non-cytotoxic results are obtained for the test substance. A good positive control would be e.g. chlorpromazine hydrochloride (50 μM) or staurosporine (200 nM). Concentrations to be applied should be tested for each cell line, as sensitivity will vary. A solvent control should also always be included. Test at least a concentration of the solvent used for the stock solution of the test substance equal to the solvent amount in the highest concentration of the test substance tested in the experiment. It is recommended to test also lower concentrations of the solvent and establish a concentration response curve.

Proper dispersion of the NM is required. Dispersion protocol needs to be optimized for each NM to be tested. The Nanogenotox protocol is a commonly applied dispersion protocol that works for many NMs and purposes (Jensen et al., 2011). As toxicity of NMs will depend upon physico-chemical properties, such as size, shape and surface coating, it is important always to perform physico-chemical characterization of the NM to be tested - both pristine material and in the actual dispersion.

Exposure with NMs and controls

At 1–16 h after seeding of the cells, they are ready to be exposed. You should use about the same time after seeding for all your experiments for consistency. Negative control, solvent control, positive control and at least three concentrations of the test substance should be applied. It is preferred to include more than three concentrations of the NM tested for establishing a concentration response curve. It is recommended to include two sets of negative controls for increased robustness of the test method. For relatively non-cytotoxic compounds, it is important to test high enough concentration to be able to conclude about the effect. For standard chemicals, the maximum concentration for non-cytotoxic compounds should not be above 5 mg/ml, 5 ml/L, or 10 mM, whichever is the lowest. The concentration range should be selected regarding expected or demonstrated cytotoxicity, solubility in the test system, changes in pH or osmolarity. For NMs, up to 100 μg/cm^2 should be used. This is equivalent to 380 μg/ml in 12-well plates (1 ml total volume) and 480 μg/ml in 6-well plates (2 ml total volume). However, for adherent cells, a dose metric per area is preferable. The highest concentration might be limited by agglomeration state of the NM to be tested.

Solvents and NMs suspension media with unknown effects should be also tested. A solvent control with the highest solvent concentration should be included in the assay. The stocks of test substances should then be prepared accordingly. The maximum solvent concentration depends on the type of solvent, but a general rule is that it should not exceed 5% for water, and 0.5% for solvents different from water or saline, (e.g. PBS and HBSS), such as methanol and DMSO.

A tip on how to choose concentrations: A linear range of concentrations (1, 2, 3, 4...) would normally be too tight, while a logarithmic range (1, 10, 100, 1000) is too much spread out. Steps of ~3-fold (e.g. 1, 3, 10, 30, 100) are often just right.

Expose the cells by adding 0.5 ml (or 1 ml for 6-well) of cell culture medium with diluted test substance (2x final concentration) or control solution, so that in total you will have 1 ml medium/well for 12-well format or 2 ml medium/well for 6-well format. Leave the cells in the incubator for colonies to form. For A549 cells, 9–12 days is sufficient. Exposure could also be stopped after 72 h by removal of medium, washing 3x in PBS and adding new medium (2 ml for 6-well plates and 1 ml for 12-well plates), however it will not be possible to wash out all the NMs as they stick to the walls of the wells and to the cells (or are taken up). Thus, for NMs it is recommended to use continuous exposure for the length of the experiment, which is until colonies clearly visible by eyes are formed. For longer exposure time points than what is mentioned above, the cell culture media could be replaced with new medium, with or without the test substance, based on the experimental setting.

Staining and counting of colonies

Colonies should be stained with 1% methylene blue. Add 20 μl methylene blue solution directly into the cell culture medium in each of the wells. Mix well by circular movements of the plate on the bench surface. Leave for minimum 30 min. The staining time can be increased if the staining is very weak. Pipette off all the medium with stain from all the wells. If needed, to reduce background staining, the plates can be rinsed carefully with water after staining but be careful not to wash off the colonies. Turn the plate upside down and leave on the bench to dry. Allow some air between the bench and the plate (e.g., place part of the lid under the edge of the plate).

Put the correct lid on each plate. Count the colonies from the bottom of the plate. Use an ink pen or a cell counter pen (e.g. e-count) to mark each counted colony to avoid double-counting. Only count colonies consisting of minimum 50 cells. Use a microscope to get familiar with selection of colonies sizes for counting. Create a template to note down the number of colonies for each well and each treatment group. Instead of manual counting, automatic counting equipment can be used (e.g. GelCount™ mammalian-cell colonies, spheroid and organoid counter, Oxford Optronix).

Results

Calculation of relative colony forming efficiency

Each viable cell will form a colony (Figure 2). After counting the colonies, the CFE value is calculated as percentage based on

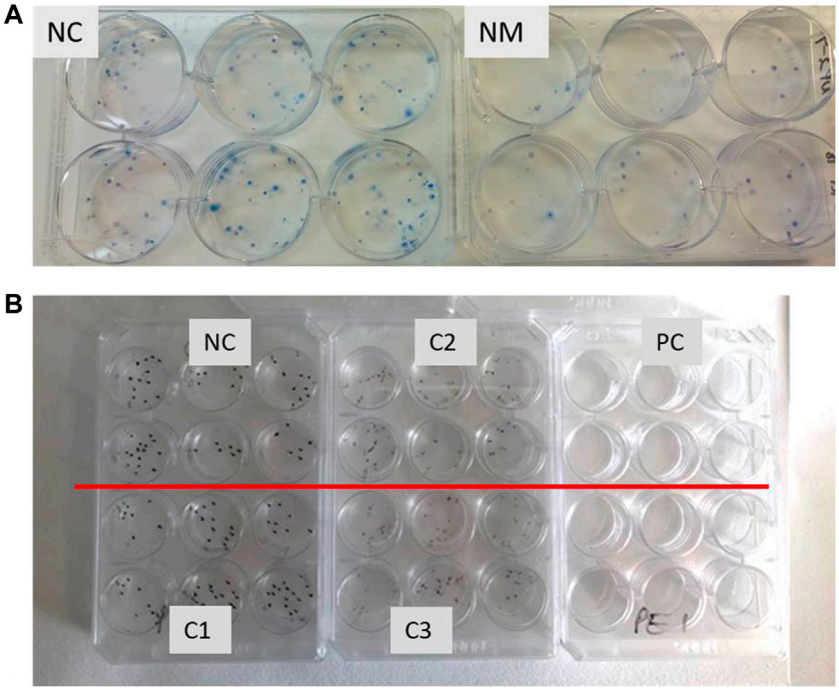

FIGURE 2
(A) Example of six well plates with cell colonies stained with methylene blue. A549 cells exposed to negative control (NC) and nanomaterial (NM), showing cytotoxic effect. Six replicate wells were exposed for each sample. **(B)** Example of 12 well plates with cell colonies stained with methylene blue. Each independent sample (negative control NC, positive control PC and tested compound with concentrations C1-low, C2-middle, C3-highest) has six parallels.

the number of colonies formed relative to the number of inoculated cells, following the formula:

CFE (%) = (colonies counted/cells inoculated) x 100.

The relative CFE (RCFE) is the ratio of viability ratio between treated cells and negative control cells. Calculate RCFE as the number of colonies in the exposed sample normalized against the negative control, by using the mean of the replicates for each treatment group:

RCFE (%) = (average number of colonies in treatment plate/average number of colonies in negative control) x 100.

In addition to the number of colonies, a reduced colony size compared with control indicate a delay in the cell cycle. Thus, it is possible to distinguish between cytotoxic effects (reduction of the number of colonies formed) and cytostatic effects (reduction in colony size).

Interpretation of results

When results are analyzed, it is important to compare with historical control data. Historical control data need to be logged for each laboratory, cell line and test method, and should include data for negative and positive controls to map baseline level for the cell line, as well as responsiveness.

Acceptance criteria for the experiments to be considered valid:

1. Exposure to the positive control must result in significant reduction (50%) or complete cell death (no colonies in the wells)
2. The plating efficiency in negative control should be comparable to historical control data for the specific cell line.

Criteria for characterizing the tested compound as cytotoxic are:

1. Cell viability (RCFE) is reduced by at least 20% compared to negative control
2. A concentration-dependent reduction in cell viability
3. Reproducible effects in at least three independent experiments

A test substance, for which the results do not meet the above criteria, is considered non-cytotoxic under the experimental conditions.

Statistical analysis could be used as an aid in evaluating the test results for example by a parametric or non-parametric statistical test for multiple comparison, such as ANOVA or Kruskal–Wallis test. This can be performed by a statistical

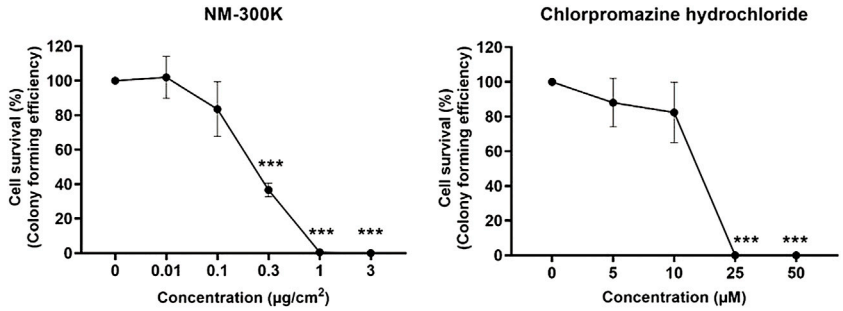

FIGURE 3
Relative colony forming efficiency (RCFE) on A549 cells exposed to nanosilver NM-300 K (JRC Nanomaterials Repository). Characterization information in El Yamani et al., 2017; Elje et al., 2020; Gábelová et al., 2017) (left image) and positive control chlorpromazine hydrochloride (right image) in 12-well plates. No colonies were seen at higher concentrations up to 75 µg/cm^2 NM-300 K or 1,000 µM chlorpromazine hydrochloride (highest concentrations now shown). Results are presented as mean ± standard deviation of two independent experiments (n = 2), each with 12 replica wells for negative control (distributed in separate plates) and four replica wells for each concentration. Significantly different effects on cell survival compared to negative control (culture medium only) were analyzed by one-way ANOVA followed by Dunnett´s post-hoc test (***$p < 0.001$) in GraphPad Prism version 9.3.1 for Windows, GraphPad Software, San Diego, California United States.

software. To compare effects between various substances it can also be valuable to calculate effect concentrations, such as EC_{50}-vaues or benchmark doses (e.g., EC_5). This can be performed by non-linear regression analysis, such as the four parameter Hill-equation.

Example of results

In Figure 3, data from our laboratory are reported as an example of typical results that can be obtained by applying the 12-well plate CFE assay for testing of NMs and chemicals (respectively silver NM-300 K by the Joint Research Centre (JRC) Nanomaterials Repository and the positive control chlorpromazine hydrochloride).

Discussion

The CFE assay is a very convenient test method for measuring cytotoxicity. It is shown to be highly compatible with testing of NMs, and free of interference since it is label-free. By miniaturizing it, the throughput is increased considerably. This makes the protocol suitable for cytotoxicity screening, although it takes some days for colonies to form. The assay is easy to perform, gives highly reproducible results, has low workload and low costs. Colonies can clearly be seen also without staining with methylene blue. However, one should note that nanoparticles can be present in the well, but those are easy to distinguish from colonies with more than about 50 cells, which is the size limit for counting. It is easy to recognize which colonies should be counted, and microscope will only be needed the first times to confirm that enough cells are present. Since the number of colonies formed is presented relative to the negative control, the most important thing is that the evaluation done with the manual counting is equal in all treatment groups, thus performed by the same person for all plates within an experiment. Ideally, the counting should be done "blinded" (with coded samples) to avoid performance bias. Quantification can also be performed by automatic colony recognition and counting. This will reduce potential bias introduced by subjective manual counting.

The test method protocol in this setting is not applicable for suspension cells, and not all adherent cells will form colonies with reasonable efficiency. Some cells are sensitive to cell density seeded and do not grow well if seeded too sparse. In our hands, the CFE assay works fine with the commonly applied cell lines A549, HepG2, JIMT-1, MDA-MB-231, T-47D and ARPE-19 (El Yamani et al., 2017; Buocikova et al., 2022, unpublished). Further, cells with exceedingly high doubling time will not be working so well with the CFE assay. For slowly growing cells and cells with low plating efficiency, the number of cells seeded should be increased, as well as the incubation time to form visible colonies. This should be tested and optimized for each cell line applied.

The seeding of cells is a critical phase for obtaining consistent results. The cells should be about 80% confluent before seeded for experiment, and the number of passages should be low (recommended below P15) to ensure high viability and avoid aging of the cell population. The cell suspension needs to be homogeneous, to ensure the same number of cells seeded in each well. It is also important to evenly spread the cells in the wells. If the variation between the wells is high, consider increasing the number of replicates. Normally, 4-6 replicate wells are sufficient.

Application of the CFE assay for toxicity testing of NMs was performed and validation was done by the JRC by interlaboratory comparison for the Petri dish format with 200 cells/dish (Ponti

et al., 2009, 2014). The CFE protocol was in our laboratory firstly validated for six well plate format (El Yamani et al., 2017; Dusinska et al., 2019), and thereafter adapted to the 12 well format. Comparable results were seen when exposing 50 cells in six well plates as with 25–30 cells in 12-well format plates (not shown). The 6-well format protocol was recently successfully applied for NMs testing with A549 and HepG2 cells (El Yamani et al., 2017; Lee et al., 2022). It is beneficial to use the 12-plate format to increase the throughput and reduce the number of plates handled during an experiment. Use of 12-well plates will also reduce the amount of NMs needed, which can be critical when the supply of particles is short. The protocol has been thoroughly tested in our laboratory with a range of different NMs, including TiO_2, SiO_2, Ag, Au, $BaSO_4$, CeO_2, ZnO, CNT, graphene NMs (El Yamani et al., 2022), and liposomes, in A549 lung cells and also in liver HepG2 cells (data not shown).

NMs should be properly dispersed to avoid aggregation/agglomeration. Some materials can be difficult to disperse in the culture medium or may have a density that does not allow them to deposit on the surface. This is, however, a challenge that will apply for all *in vitro* models, which depend on exposures under submerged conditions. Cell-particle interaction should be assessed in any toxicological endpoint, especially when negative results are obtained.

Other cells found to be compatible with the assay are for example different human breast cancer cells and V79. The 12-well format protocol was standardized for application with NMs and validated by interlaboratory comparison by four laboratories in three different countries within the H2020 NMBP-13 RiskGONE project (paper in preparation). It is essential that the laboratory performing the test establishes historical control values for negative and positive controls for each cell line applied. Acceptance criteria for the test methods should be set based on historical control data, and plating efficiency and effects should generally be within mean ± 3 times the standard deviation, calculated from the historical control data for the cell line. Too large inaccuracy in cell seeding giving high variation in cell number seeded in the different wells, will introduce high variability in the RCFE values calculated. Since cell viability after treatment is calculated relative to negative control, it is of importance to have proper values for this. Thus, for more robustness, it is recommended to include two negative control plates in case one of them fails.

Exposure time can be continuous for the length of the experiment or stopped earlier. Ponti et al. (2014) reported exposure for 72 h and replaced the exposure medium with fresh culture medium. However, it is not possible to wash out all particles, as they normally stick to the plastic as well as to the cells, so continuous exposure is preferred.

It is recommended to calculate the effective concentration giving 50% reduction in cell viability (EC_{50} values) for better categorization of toxic potency of the test compounds.

It is important to report data in a harmonized and FAIR way (Jeliazkova et al., 2021). For several assays, including the CFE assay, data collection templates (with a function for automatic calculation of the results from the reported raw data) were developed within the RiskGONE project. The template is available upon request through the eNanomapper database, and it will be made publicly available. In this way, data from different laboratories can be compared and data can be used for meta-analyses.

The CFE assay is a sensitive assay for detection of cytotoxic effects, and as it is non-colorimetric and non-fluorescent it is especially applicable for testing of NMs to avoid interference between the NM tested and the readout or reagents of the assay, which is commonly seen with colorimetric or fluorometric assay e.g. the MTT, and other assays. Unlike most cytotoxicity assays which have an exposure time of less than 48 h, the CFE assay can be regarded as a sub-chronic assay since the exposure time is for several days, most often about 10 days. The CFE assay reflects true viability, i.e., the capacity of cells to proliferate. Thus, in the CFE assay, direct toxic effects on each cell are determined.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

ER-P and MD conceptualization. ER-P, NE, and MD were involved in planning. ER-P and EM adapted the SOP for the 12 well format. ER-P performed the experimental work and planning, the data analysis, and drafted the manuscript. EM, EE, EL, MD and NE contributed to the manuscript writing. MD supervised the work. All authors discussed the results and the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The alamar blue assay in the context of safety testing of nanomaterials

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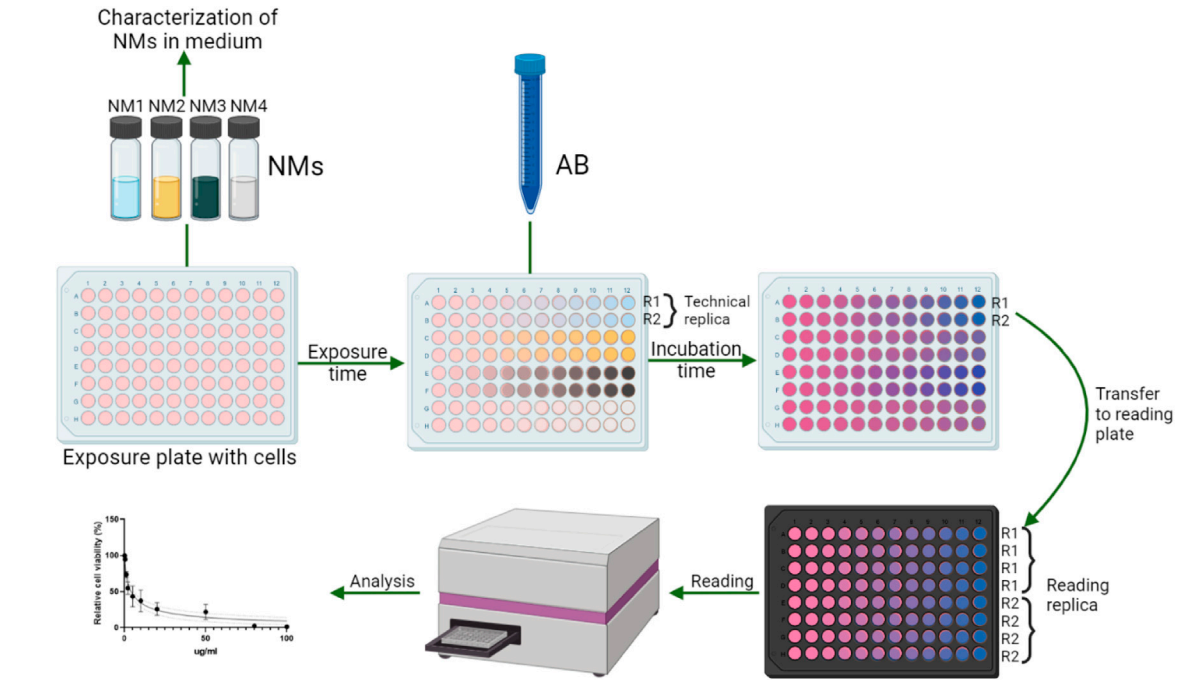
The Alamar Blue (AB) assay is widely used to investigate cytotoxicity, cell proliferation and cellular metabolic activity within different fields of toxicology. The use of the assay with nanomaterials (NMs) entails specific aspects including the potential interference of NMs with the test. The procedure of the AB assay applied for testing NMs is described in detail and step-by-step, from NM preparation, cell exposure, inclusion of interference controls, to the analysis and interpretation of the results. Provided that the proper procedure is followed, and relevant controls are included, the AB assay is a reliable and high throughput test to evaluate the cytotoxicity/proliferation/metabolic response of cells exposed to NMs.

KEYWORDS

alamar blue, cytotoxicity, nanoparticles, nanomaterials, viability, cellular metabolic activity

1 Introduction

Cytotoxicity is one of the main endpoints to be assessed in any toxicological investigation. There is a wide range of methods that can be used to investigate the cytotoxic effects of chemicals and other test substances, including nanomaterials (NMs). These methods are based on diverse principles and cell functions, e.g., membrane integrity (assays such as trypan blue exclusion, neutral red uptake, LDH release), relative cell growth (measuring the number of cells in the population, reflecting cell death together with changes in cell proliferation), ability to survive and form colonies [colony forming efficiency assay (CFE)], and cellular metabolic competence (Riss et al., 2016; 2019; Méry et al., 2017). This last class of methods uses the cellular metabolic activity to measure viability or proliferation in a cell population. Metabolically active cells maintain a reducing environment within their cytosol. This is taken advantage of through the use of colorimetric or fluorometric redox indicators, and their conversion that can be measured spectrophotometrically. Together with tetrazolium salt-based assays such as MTT and WST-1, the Alamar Blue (AB) assay is one of these metabolism-based methods. Since its release in 1993, the AB assay has become widely used to investigate *in vitro* the cytotoxicity of various test compounds, and the proliferation of cell lines, bacteria and fungi (Fields and Lancaster, 1993; Ahmed et al., 1994). AB is based on the fluorometric



GRAPHICAL ABSTRACT

redox indicator resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), a blue-colored non-fluorescent compound. After intracellular uptake, the oxidized resazurin is reduced to the fluorescent resorufin (7-hydroxy-3H-phenoxazin-3-one) due to the reducing environment of the cytosol in the cells. The conversion of resazurin to resorufin is mediated by intracellular diaphorases, with NADPH or NADH as reductant (O'brien et al., 2000). Resorufin produces bright red fluorescence, with excitation range of 530–570 nm and emission range of 580–610 nm, that can be quantified (fluorescence intensity) and used as a measure of cell viability. The test can also be read on the basis of the absorbance at 570 nm, using 600 nm as a reference wavelength (the values need to be normalized on the reference wavelength).

Resazurin (and resorufin) is water-soluble, stable in culture medium, non-toxic and permeable through cell membranes, and the AB assay has proved to be robust, simple to perform and relatively cheap, thus presenting many advantages compared with other cell viability and proliferation assays (Rampersad, 2012). As an example, the AB assay has clear advantages with respect to the MTT assay, another common cytotoxicity method based on cellular metabolic activity: 1) First, being water-soluble resorufin is released in the cell culture medium, which can be directly used for measurement. In contrast, the insoluble formazan crystals produced by the conversion of the tetrazolium salt in MTT need to be dissolved by a solubilization step before reading the test. 2) Additionally, the cells used for MTT will thus be destroyed during the solubilization step, while the cells used for AB can be employed for other purposes. 3) AB is non-toxic, while MTT has been reported to be cytotoxic itself (Ghasemi et al., 2021). 4) Finally, AB has been reported to be more sensitive at detecting cytotoxicity than the MTT assay (Hamid et al., 2004).

The AB assay can be used in a high throughput set up, allowing screening of the toxicity of a large number of compounds at the same time (Hamid et al., 2004; OECD, 2018), and it has been widely used within the field of nanotoxicology [287 PubMed search results with keywords: (Alamar Blue) AND (nanomaterials OR nanoparticles)].

For cytotoxicity testing of NMs, interference is an important challenge especially in relation to colorimetric and fluorescent test methods. NMs can in general interfere with toxicological tests at different levels, from the assay's chemical reactions to the test readout (Rampersad, 2012; Guadagnini et al., 2015). As an example, spectroscopic analyses have highlighted interactions (indicated by reduction of absorption/fluorescent emission) of single walled carbon nanotubes (SWCNT) with several dyes used for cytotoxicity investigations, including Neutral Red, MTT, WST-1, and also AB, which was found to be the most sensitive and reproducible method (Casey et al., 2007; Davoren et al., 2007). Interference issues might account for the inconsistency sometimes found in the responses obtained with the different cytotoxicity methods; therefore the verification of the results by the use of at least two methods is recommended

TABLE 1 Information on the NMs used.

NMs	Provider and code	European registry of materials (van Rijn et al., 2021)	Particle size according to the provider
TiO_2	JRCNM01005a	ERM00000064	15–24 nm
ZnO	Sigma Aldrich, 721077	ERM00000063	<100 nm (TEM)
GEMNS-IVA1	Graphene-Encapsulated Magnetic Nanoparticles functionalized with polymers (PEI, 25 kDa) and decorated with human IgG	NA	Full characterization available in (Kasprzak et al., 2016)

(Worle-Knirsch et al., 2006; Dusinska et al., 2015, 2017). Repeated washing steps should be performed after exposure to remove as many particles as possible. However, internalized particles or particles adhering to the cell surface will not be removed after washing (Davoren et al., 2007). To properly address this issue, appropriate controls to check for interference should always be included in the experiments.

General considerations and potential pitfalls of the AB assay have been previously reported in the literature (Rampersad, 2012). Here we thoroughly describe the AB procedure applied to NMs, present examples of results obtained with various types of NMs and discuss possible interpretation of the results. The method here described has been refined during the many years of NM-related research in our laboratories, and within the H2020 project RiskGONE, whose core aim is to evaluate the suitability of various *in vitro* tests for reliable hazard assessment of NMs, and to deliver sound protocols that have been adapted for use with NMs.

2 Materials and equipment

2.1 Reagents and materials

AB can be purchased from different providers as a ready-to-use solution, although a resazurin sodium salt is also available. The procedure below is for the ready-to-use solution, and it was developed in our laboratory based on the manufacturer's instructions, with the addition of some refined steps and specific measures for testing NMs. Further adaptations might be needed by the operator according to the product purchased. The manufacturer's instructions should always be the basis on which to apply the NM-specific measures.

Over the years, several NMs have been tested with the AB assay in our laboratory within different projects. Here we mostly refer to the work performed under the ongoing H2020 RiskGONE project, where the AB assay was critically examined for its suitability for testing NMs. To this end, different NMs were selected. As an example, here we present the results obtained with TiO_2-based NMs JRCNM01005a [European Registry of Materials: ERM00000064 (van Rijn et al., 2021)] and ZnO NMs from Sigma Aldrich (supplier code 721077, ERM00000063). Results obtained on NMs tested within the EuroNanoMed II project GEMNS (GEMNS-IVA1) are also reported. More information on the NMs is reported in Table 1.

2.2 Equipment

Equipment needed for the AB assay include a laminar flow hood, light microscope, automated cell counter/Bürker chamber, pipettes, CO_2 incubator, refrigerator, water bath, vortex, autoclave.

For reading a spectrometer, fluorometer, or plate reader for higher throughput, with filters or monochromator to read fluorescence within excitation range of 530–570 nm and emission range of 580–610 nm, or absorbance at 570 and 600 nm are needed for reading. In this manuscript a microplate reader FLUO star OPTIMA was used to read fluorescence (excitation 530 nm, emission 590 nm).

3 Methods

3.1 Cell culture conditions

The AB test can be applied to both adherent cells and cells in suspension, as well as co-cultures and 3D advanced models. The cells are cultivated according to the model's specific needs.

Different exposure plates can be used. The use of 96 well plates is convenient to increase the throughput of the method, especially useful for toxicity screening.

The number of cells to be seeded for exposure is an important parameter to consider, and it should be adjusted based on the cell type used. It has been reported that the cell density at the moment of exposure (confluency) can affect the sensitivity of the cells to NMs; i.e., lower EC50 values are observed when fewer cells are exposed, in tetrazolium based assays (Geys et al., 2010; Heng et al., 2011; Elliott et al., 2017). This has been observed also in our laboratory with the AB assay (the data are not shown, as further investigations on this topic are needed). According to the OECD Guidance Document on Good *In Vitro* Method Practices (GIVIMP) (OECD, 2018) a fixed and pre-determined seeding density should be used to improve consistency across

experiments, and can contribute rather than an estimation of the cell confluency that is prone to error and contribute to variability in baseline cell physiology. Thus, the seeding density is a parameter that needs to be harmonized within and among laboratories. It is generally recommended that the cells should be used in the exponential growth phase (Rampersad, 2012).

The cell lines and conditions used in this study are reported below.

3.1.1 Adherent cells

The human lung epithelial cell line A549 was maintained in DMEM medium supplemented with 9% FBS and 1% penicillin/streptomycin, in an incubator at 37°C, 5% CO_2; the day before exposure, 1.5×10^4 cells/well were seeded in a 96 well plate. The human bronchial epithelium cell line BEAS-2B was cultivated in LHC-9 medium without supplements; the day before exposure, 2.0×10^4 cells/well were seeded in a 96 well plate.

3.1.2 Suspension cells

The human lymphoblast cell line TK6 (suspension cells) was maintained in RPMI medium supplemented with 9% HS and 1% penicillin/streptomycin; the same day of exposure, 1.5×10^4 cells/well were transferred in a 96 well plate.

N.B. Cells in suspension can be transferred to the exposure plate on the same day of exposure. Cells must be seeded in half of the medium volume that will be used for exposure, e.g., if 200 µl of medium per well are used in the 96 well format plate as final exposure volume, the correct number of cells should be transferred in 100 µl of medium per well.

3.2 Nanomaterial dispersion and characterization

The NMs used for the test should be properly dispersed and characterized. Different approaches and methods are available to address the NM dispersion. Not all NMs respond equally to the same handling. In this work we mainly used the protocol described in (Deloid et al., 2017). When satisfactory dispersion was not obtained (particle size and size distribution by DLS analyses vs. expected particle size based on the provider's declaration), the NANOGENOTOX protocol was tested and applied if better results were observed.

Proper NM characterization should always accompany any toxicological study.

3.3 Exposure conditions and treatment with test substance and controls

For exposure of cells to the test substance, it is good practice to have technical replicates within the same experiment e.g., at least two wells exposed to the same treatment. At least three independent experiments should be performed.

A negative control (NC) and a positive control (PC) must always be included, i.e., cells unexposed to the test substance (maintained in the cell culture medium) and cells exposed to a known cytotoxic agent, respectively. This allows assessment of the performance of the assay. A possible positive control recommended for the AB assay, and used in the experiments here reported, is chlorpromazine at 50 µM. Other agents giving a positive response, to be adapted to the cell model used, can be considered. Specific considerations and guidance on the selection of proper positive control materials for *in vitro* assays can be found in the literature (Petersen et al., 2021).

A range of NM concentrations should be included to establish a concentration response curve. A minimum of 3 concentrations in addition to the negative control sample should be considered. Cell-particle interaction should be assessed, i.e., NM deposition or internalization.

> N.B. Cells in suspension must be exposed by adding twice concentrated exposure doses at ratio 1:1 to the seeding medium volume; e.g., if 200 µl of medium per well are used in the 96 well format plate as final exposure volume, 100 µl per well of 2x concentrated exposure suspensions must be added to 100 µl of plated cells.

Interference controls for NMs must be included. These consist of NMs in cell culture medium in wells without cells (only medium with NMs). The highest concentration tested for the NMs should be included as a minimum condition; however other test concentrations could be added in an optimal situation. These controls will be incubated for the same time as the exposed cell samples.

3.4 Alamar Blue preparation

The AB storage conditions should be followed according to the manufacturer's instructions. In general, the AB solution should be stored in the dark and protected from light during the performance of the assay, since the compound is light sensitive. The AB solution can be stored at room temperature or at 4°C (for extended shelf life); if stored cold, it should be equilibrated to room temperature before use.

The solution should be slightly shaken to ensure all components are completely in solution before use.

> N.B. Clogging or precipitates of AB can be sometimes observed. Clogging in the staining solution will affect the results, making them unreliable. It is important to always check that the staining solution is free of precipitates. These can be removed by filtering the AB solution or the staining solution (AB and medium) before mixing it with the medium (syringe-filter through a 0.2 or 0.45 µm pore filter).

3.5 Alamar Blue incubation

As the AB assay is very sensitive, precise pipetting is important in the following steps, to obtain reliable results. Do not leave any residue of PBS or medium after washing, to avoid diluting the staining solution. Pipette the exact volume of the staining solution into the wells. Uneven volume pipetting will render results unreliable. Special attention should be given when using multichannel pipettes. With adherent cells, gently pipet along the wall of the well, to avoid detaching the cells by harsh pipetting.

3.5.1 Adherent cells

- At the end of the exposure to the test substance or NMs, prepare the staining solution by adding 10% AB to fresh cell culture medium pre-heated to 37°C.
- Exposed cell samples: Remove the exposure medium and wash the cells twice in PBS or medium. Add the staining solution, e.g., 200 µl in the 96 well plate.
- Interference controls for NMs: add pure AB to the interference controls to a final concentration of 10%; e.g., if 200 µl of medium are used in the 96 well plate, mix the medium in the interference control wells, discard 20 µl of medium and replace with 20 µl of pure AB (alternatively add 22 µl of pure AB to 200 µl of medium). Mix thoroughly again.

3.5.2 Suspension cells

- At the end of the exposure to the test substance or NMs, add pure AB to the exposed cells samples and interference controls to a final concentration of 10%, e.g., if 200 µl of medium are used in the 96 well plate, mix the medium in the wells, discard 20 µl of medium and replace with 20 µl of pure AB (alternatively add 22 µl of pure AB to 200 µl of medium). Mix thoroughly again.

3.5.3 All cell types

Always include blank control sample: add the staining solution (10% AB in cell culture medium) in empty wells (wells without cells). Use the same volume as the other samples, e.g., 200 µl in the 96 well format plate.

After adding AB, incubate the plate for 1–4 h at 37°C, 5% CO_2, until a change in AB color can be observed. A longer incubation time may be used for higher sensitivity. The incubation time depends on several factors which include the cell type (cells with different metabolism convert AB at different speed rate), the cell model (3D models e.g., spheroids might take more time to convert AB with respect to the same cell type in 2D condition, due to the reduced cell surface available, thus reduced AB uptake) and the number of seeded cells (Bonnier et al., 2015). Find the optimal incubation time for the system used and standardize it for further experiments. In our experience, 3 h incubation time is appropriate in most cases.

3.6 Reading

At the end of the incubation time, the AB signal can be read in absorbance or fluorescence by a spectrometer or fluorometer, respectively. Fluorescence seems to provide higher test sensitivity compared to absorbance. The staining solution can be transferred to reading supports such as cuvettes or, to increase the throughput of the assay to, e.g., 96-well format reading plates. For reading of the fluorescent signal in microplates, black plates should be used, as in the transparent plates signal interference from the next wells might occur.

To increase the robustness of the results, 3 or 4 reading replicas should be prepared from each sample. For example, if 96 well plates are used for exposure, transfer 40 µl of staining solution (medium) 4 times into 4 different wells of flat bottom 96 well black polystyrene microplates.

> N.B. The presence of bubbles in the medium during reading can affect the results, and thus must be avoided. Pipetting when transferring the medium to the reading support must be done with care; the reverse pipetting technique might be of help for this. After pipetting, the presence of bubbles should be checked by visual inspection. There are several ways to remove bubbles, e.g., blowing a gentle stream of air or ethanol vapor over the plate, putting the plate or cuvette under vacuum, or using a 10 µl pipette tip (Petersen et al., 2022). The potential application of a bubble control might be considered (ISO, 2018; Petersen et al., 2022).

The fluorescence signal or absorbance can be read at appropriate wavelengths in the microplate plate reader. For fluorescence, the AB excitation range is 540–570 nm and emission range is 580–610 nm. The AB absorbance can be read at 570 nm, using 600 nm as a reference wavelength. The data are obtained as fluorescence units (FU) or optical density (OD), respectively.

3.6.1 Optional

A centrifugation step can be included before transferring the staining solution to the reading plate/support. Preliminary data in our laboratory suggest that this step could be especially useful when suspension cells are used, or in case of interference of NMs with the test reading (data not shown).

3.7 Data analysis

The results of the AB assay can be presented as relative fluorescence (or absorbance) intensity (percentage) of the exposed samples towards that of unexposed cells i.e., the negative control. While results are linear and quantitative for both fluorescence and absorbance, the fluorescence readings provide higher sensitivity.

TABLE 2 Historical PC data for Chlorpromazine 50 μM (by cell lines and exposure time). Data are reported as relative fluorescence intensity (%) with respect to the NC (100%).

Cell line, exposure time	Average ±SD	Minimum value	Maximum value
A549, 3 h	3.9 ± 7.7	−3.1	18.5
A549, 24 h	−1.8 ± 3.8	−11.3	1.7
BEAS-2B, 3 h	2.0 ± 9.9	−8.2	20.6
BEAS-2B, 24 h	−1.4 ± 1.7	−4.5	0.2
TK6, 3 h	56.6 ± 15.1	42.5	77.9
TK6, 24 h	4.4 ± 8.9	16.1	−4.9

The values for the fluorescence reading are calculated as described below:

- calculate the average of the reading replicates
- subtract the average of the blank control (samples with AB and without cells) from all the data
- calculate the average of the negative control samples (technical replicate)
- calculate the relative fluorescence intensity as the ratio between the exposed samples and the average of the negative control samples, and express it as a percentage according to the equation:

Relative fluorescence intensity: FU exposed sample/FU average negative controls *100.

The average ±SD (or SEM) of at least 3 independent experiments should be calculated and reported as the final result.

3.7.1 Interference controls for nanomaterials

Interference control samples for NMs (10% AB in medium + NMs without cells) should be compared with the blank control samples (10% AB in medium without cells). This can be done at the level of FU, i.e., no significant difference between FU of interference control samples and FU of blank samples indicating lack of interference. Alternatively, the interference controls can be analyzed as the other samples, i.e., the relative fluorescence intensity can be calculated. In this case the relative fluorescence intensity value obtained for the interference control sample is expected to be around 0%. This last approach has been used for the results here reported.

3.7.2 Historical positive and negative controls

It is highly recommended to build up an historical control database, with both negative as well as positive controls for each cell type and time point investigated. This allows the laboratory to demonstrate the ability to perform the assay consistently, and to show that the cells are capable of picking up positive effects and have reasonably low variability in responses. When reporting the results, it is advisable to show the average and minimum-maximum values of negative/positive historical controls from the last 10–20 experiments performed in the laboratory.

In the AB assay the results are normalized over the NC, so this will always be 100% (Relative fluorescence intensity). Historical PC values from our laboratory (Chlorpromazine 50 μM, on different cell lines and exposure times) are reported in Table 2 as an example (average of the last 10 experiments ±SD, minimum and maximum values).

3.7.3 Data collection templates

Within the RiskGONE project, a data collection template has been developed for AB, to move towards data reporting harmonization and data FAIRness. The template provides a function for automatic calculation of the results from the reported raw data. The template is available upon request through the eNanomapper database, and it will be made publicly available (Jeliazkova et al., in preparation).

3.8 Interpretation of the results

The criteria for determining if a test compound is cytotoxic can depend on the application field.

In general, the test substance is considered cytotoxic if all the following conditions occur:

- The signal in the cells treated with the test substance is reduced at least by 20% compared to the negative controls (untreated cells)
- A concentration-related reduced signal is observed
- The results are reproducible, i.e., at least 3 independent experiments confirm the results

The first point reflects the fact that the biological relevance of the results needs to be considered. According to the historical controls, the variability (calculated as standard deviation, SD) between the experiments can account for around 10% of the relative fluorescence intensity calculated with respect to the NC. To ensure the biological relevance of the observed reduction, 2x SD (20%) is selected as a threshold to state that a compound is

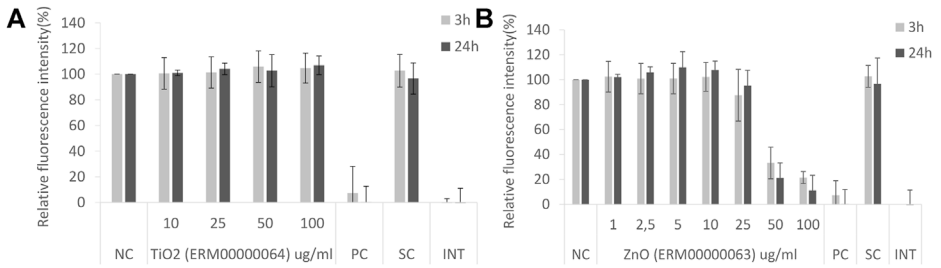


FIGURE 1
Alamar Blue assay on A549 cells after 3 and 24 exposure to nanomaterials: **(A)** TiO_2 JRCNM01005a (ERM00000064) and **(B)** ZnO NMs from Sigma Aldrich (supplier code 721077, ERM00000063). NC-negative control, PC-positive control, SC-solvent control, INT-interference control.

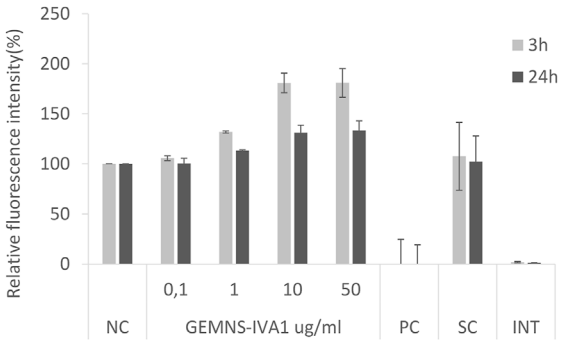


FIGURE 2
Alamar Blue assay on A549 cells after 3 and 24 exposure to GEMNS-IVA1 nanomaterial. NC-negative control, PC-positive control, SC-solvent control, INT-interference control.

cytotoxic. Statistical methods are used as an aid in evaluating the test results. However, the statistical significance will not be the only determining factor for cytotoxicity.

A test substance, for which the results do not meet all the above criteria, is considered non-cytotoxic under the assay conditions.

Positive results in an *in vitro* cytotoxicity test indicate that the test substance induces a cytotoxic effect in the cultured cells used. Negative results indicate that, under the test conditions, the test substance does not induce cytotoxicity in the cells used.

3.8.1 Interpretation of the interference controls results

If the FU value of the interference samples significantly deviates from that of the blank sample (or the relative fluorescence intensity deviates from 0%), it means that the NMs interfere with the AB assay and the results obtained are not reliable. Also in this case, the criteria for determining if an interference is present can depend on the application field, and on the criteria applied to determine the cytotoxicity of the test compound. A statistically significant difference between the interference control and the blank could be a sufficient indication of interference.

4 Results

Here we report some examples of results obtained with different NMs used in various projects. Within the ongoing H2020 RiskGONE project, the cytotoxicity/cellular metabolic activity of cells exposed to different NMs was analyzed by the AB assay, to evaluate the method's suitability for testing NMs. Both adherent (A549, Figure 1) and suspension cells (TK6, data not shown) were used to test the assay. The interference controls were also included and the reported value were similar to the blank for all the NMs tested (no interference of the tested NMs with the AB assay was detected, Figure 1).

Non-cytotoxic compounds such as TiO2 ERM00000064 result in a relative fluorescence intensity similar to that of the negative control (100%) as shown in Figure 1A. In the case of a cytotoxic compound such as ZnO ERM00000063 (Figure 1B), a concentration response curve will be obtained, showing the reduction of signal with increasing concentrations of NMs. The shape and the slope of the curve give an indication of the severity of the toxicity of the test substance. From this slope, the EC50 (effective concentration producing 50% of the maximal response) can be calculated.

Besides the more common situations reported above, different results can be observed. Interference of NMs with the test could be found, although in our experience it was rarely detected. An increase of the relative fluorescence was observed on a few occasions in the samples exposed to the test substance compared to the negative control. NMs tested within the EuroNanoMed II projects GEMNS highlighted a concentration-dependent increase of the AB signal. This effect was observed in both A549 cells (Figure 2) and BEAS-2B cells (data not shown). In both cases the effect was more evident after 3 h exposure to the NMs, compared to 24 h exposure. An

interference effect was ruled out as shown by the interference control (Figure 2).

5 Discussion

The AB assay is widely used on mammalian cells and cell lines, bacteria and fungi to establish the relative cytotoxicity of test substances, demonstrating it to be a reliable test. However, there are important aspects (and possible disadvantages) in this assay to take into consideration, the first being that AB is not a direct cell counting technique, and the fluorescence or absorbance signal can be affected by changes both in the number of living cells and in cellular metabolism. The test provides information at the level of the whole cell population, not the single cell, with the assumption that cytotoxicity will determine a reduction in the number of cells, and a lower resorufin signal. Damaged and/or non-viable cells have lower innate metabolic activity and thus generate a proportionally weaker signal than healthy cells. On the other hand, some compounds, including NMs, can increase the metabolism of the cells (Kladko et al., 2021), which will result in a higher AB signal. Besides, alterations of cell proliferation are not always accompanied by cell death. The test substance might influence cell proliferation, either slowing it down or accelerating it, affecting the total number of cells, and thus the test results.

For this reason, caution should be taken in the interpretation of the data, in the sense that the results might not be directly an indication of cytotoxicity, but an effect of the cellular metabolic activity or proliferation. These processes, cytotoxicity, metabolic activity and proliferation, all play together in determining the response of the test, thus making the interpretation of the results more challenging.

Interestingly, it has also been reported that a further reduction of resorufin leads to the formation of colorless and non-fluorescent products. Thus, aberrant results might be generated when healthy cells over-reduce the AB producing a weaker signal compared to less active or dying cells (O'brien et al., 2000).

In this context, the use of multiple assays (at least two cytotoxicity tests) is recommended to reduce false negative/positive results (Worle-Knirsch et al., 2006; Dusinska et al., 2015, Dusinska et al., 2017, Azqueta et al., 2022). Non-colorimetric assays such as the CFE or clonogenic assay, and impedance-based assays represent valuable and interference-free tools to support the cytotoxicity investigation of NMs (Herzog et al., 2007; Cimpan et al., 2013; Rundén-Pran et al., this special collection). Visual (microscopy) evaluation of the status of the cells should also always be performed.

In the use of *in vitro* tests with NMs, one should take into consideration the different behaviors and physico-chemical properties of these materials compared to chemicals in general. Just to mention a few, the capacity of NMs to adsorb other compounds on their surface, and optical properties such as optical density that can interfere with the transmission of light, and in some cases autofluorescence. These properties are in particular relevant when colorimetric or fluorometric test methods are used, such as the AB assay. As this test is based on the development and measurement of fluorescence (or alternatively absorbance), there is a chance for NMs to interfere with the test read-out. In addition, NMs might directly interact with the reagent, altering its structure and affecting the normal reactions that should occur.

It is not the aim of this work to deeply investigate the possible interference of the AB assay with NMs, but rather to revise and adapt the method for application to this class of substances. Interference controls are meant to assess any interference of the NMs with the AB. This can happen at different levels, from interactions with the reagent or product at different steps of the assay, or with the reading as quenching of fluorescence or as a false induced signal, e.g. autofluorescence. In our approach, possible interference is investigated by mixing the NMs with the AB and analyzing the signal. This sample can be analyzed as the other samples, i.e., the relative fluorescence intensity can be calculated. In this case the expected outcome is a null relative fluorescence intensity, similar to the blank samples. A higher signal could indicate e.g., autofluorescence of the NMs analyzed, while a lower value could indicate shading of the fluorescent signal. However, this last condition would be difficult to detect with this setting of interference control, as resazurin alone is not fluorescent. An additional control mixing NMs with the fluorescent resorufin could be considered.

The interpretation of the results obtained from cells exposed to NMs and chemicals in general can sometimes be tricky. On a few occasions we observed an increased relative fluorescent signal in cells exposed to NMs compared to the non-exposed (negative) control, such as is shown here in Figure 2. Possible reasons for this effect could be an increased metabolism of the cells in response to the test substance, or increased proliferation resulting in a higher number of cells. However, the first hypothesis seems more likely in our case, at least for the effect observed at 3 h after exposure, which might be too early to see an increased number of cells. This last option might explain the slight increase of fluorescent signal at 24 h when the cells had time for the cell cycle to be completed.

In general, an interference of the NMs with the test cannot be excluded in case of increased fluorescent signal, e.g. due to NM autofluorescence, or to NMs reacting with the resazurin in the AB and reducing it to the fluorescent resorufin. Strongly reducing NMs may directly reduce resazurin non-enzymatically. Compounds that trigger the release of superoxide can cause reduction of resazurin by superoxide. This may result in a false cytotoxicity outcome. In our case here this seems to be excluded as the interference controls did not show any increased signal.

In case of interference of NMs with the test (or even when suspension cells are used), centrifugation of the samples before reading might be a way to remove the NMs (and cells in suspension). A plate spinner helps in case of the high throughput setting. Another possible solution suggested is to use the interference controls values as background value, and thus subtract them from the correspondent NM-exposed sample value (Guadagnini et al., 2015; Ciappellano et al., 2016). In this case, interference control samples for all the NM concentrations tested must be included, and the relative value subtracted for each concentration.

The AB assay is included as a part of the OECD TG 249 for the RTgill-W1 fish cell line acute toxicity test into 24-well plates. No dedicated standard method with detailed operating procedure is available at the moment, e.g., for the use with other cell lines, for higher throughput format, and for testing with NMs. The work here presented will help the standardization of this test to support sound safety assessment of NMs, by providing a detailed procedure that can be tested among different laboratories. The next steps towards standardization should include a validation of the procedure through interlaboratory testing of specific settings (e.g., selected cell lines and test materials) to demonstrate the robustness of the method, i.e., the repeatability of the responses to standard NMs. As a further step in this direction, we here reported the results obtained on widely used cell lines, such as A549, exposed to some easily acquired NMs, and unequivocally identified through the newly proposed European Registry of Materials (van Rijn et al., 2021). Eventually, the standardized and validated procedure might be submitted to the OECD as a standard project submission form (SPSF).

In conclusion AB is a reliable test to evaluate the cytotoxicity/proliferation/metabolic response of cells exposed to NMs. Being high throughput makes it an ideal tool to be used on a large scale and in parallel or in combination with other assays e.g. the comet assay for genotoxicity (Azqueta et al., 2022). However, washing steps after exposure and proper controls for possible interference of the NMs with the test need to be always included. The coupling of this metabolism-based test with another class of cytotoxicity method based for example on membrane integrity or cell number is also a major recommendation to strengthen the results.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

EL wrote the manuscript and performed the experiments. NE, ER-P, and MD adapted the SOP for the NMs testing. All authors reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The miniaturized enzyme-modified comet assay for genotoxicity testing of nanomaterials

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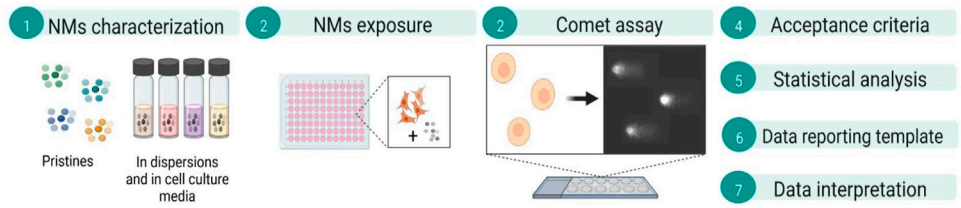
The *in vitro* comet assay is a widely applied method for investigating genotoxicity of chemicals including engineered nanomaterials (NMs). A big challenge in hazard assessment of NMs is possible interference between the NMs and reagents or read-out of the test assay, leading to a risk of biased results. Here, we describe both the standard alkaline version of the *in vitro* comet assay with 12 mini-gels per slide for detection of DNA strand breaks and the enzyme-modified version that allows detection of oxidized DNA bases by applying lesion-specific endonucleases (e.g., formamidopyrimidine DNA glycosylase or endonuclease III). We highlight critical points that need to be taken into consideration when assessing the genotoxicity of NMs, as well as basic methodological considerations, such as the importance of carrying out physicochemical characterization of the NMs and investigating uptake and cytotoxicity. Also, experimental design—including treatment conditions, cell number, cell culture, format and volume of medium on the plate—is crucial and can have an impact on the results, especially when testing NMs. Toxicity of NMs depends upon physicochemical properties that change depending on the environment. To facilitate testing of numerous NMs with distinct modifications, the higher throughput miniaturized version of the comet assay is essential.

KEYWORDS

alkaline comet assay, nanomaterial, genotoxicity, DNA damage, interference, lesion-specific endonucleases, oxidized DNA bases, 12 mini-gels



GRAPHICAL ABSTRACT

1 Introduction

A recent paper in the journal Nature Protocols describes in detail the various protocols for the *in vitro* comet assay (Collins et al., 2022). Here we focus on the *in vitro* testing of nanomaterials (NMs) using the alkaline comet assay based on 12 mini-gels per slide, in combination with lesion-specific endonucleases.

We address the most relevant points to be taken into consideration when assessing NM genotoxicity. Hazard assessment of NMs with conventional methods for chemical testing poses a challenge, owing to physicochemical properties of NMs, such as optical features, reactivity, and surface area, which differ from those of the corresponding bulky chemicals. NMs may interfere with the test assay endpoints, especially those relying on colorimetry or fluorimetry principles, leading to potentially biased data (Guadagnini et al., 2015; Karlsson et al., 2015).

The particular physicochemical properties of NMs may lead to potential interference with standard test methods including the comet assay. Certain NMs, such as TiO_2, and nanogold, are especially likely to cause interference. The possibility of interference with the comet assay by NMs has been discussed previously (Kain et al., 2012; Magdolenova et al., 2012; Karlsson et al., 2015; Di Bucchianico et al., 2017; George et al., 2017). Interference may happen either directly or indirectly: 1) direct/physical interference of the NMs with the DNA (after lysis) creating additional breaks or adducts; 2) possibilities for NMs to interfere by reducing or blocking the DNA migration during electrophoresis; 3) inhibition/interaction with Fpg activity; 4) quenching/autofluorescence during quantification of signals/scoring; 5) interference of photosensitive particles with direct light may cause changes in the particles (e.g., increase their reactivity or effect). We therefore suggest here that proper interference controls should always be included in the experimental design.

The effect of DNA damaging agents can be detected by a wide range of toxicology assays. The single cell gel electrophoresis (or comet assay), is widely used for detection of DNA damage induced by chemicals, and is the most used method for testing NMs (Magdolenova et al., 2012; Huk et al., 2015a; Magdolenova et al., 2015; El Yamani et al., 2017; Garcia-Rodriguez et al., 2019). The alkaline comet assay measures DNA damage (single and double strand breaks and alkali-labile sides) in eucaryotic cells (Collins, 2004; Collins et al., 2017a; Collins et al., 2017b; Gajski et al., 2019; Collins et al., 2022). Since it was introduced in 1984 (Ostling and Johanson, 1984), the assay has gone through several modifications to increase sensitivity and reduce variability, as well as to increase its robustness and applicability in different areas. While the *in vivo* comet assay has been validated, and OECD Test Guideline (TG 489) approved (OECD, 2014b), there is not yet any OECD test guideline for the *in vitro* comet assay. The protocol for testing NMs by the *in vivo* comet assay is described by Elsepuru et al. (2022).

The *in vitro* comet assay has been miniaturized to allow many more samples to be analysed in a single experiment. Thus, 12 mini-gels are applied to one slide instead of the one or two gels as in the original procedure; or 96 mini-gels can be placed on a GelBond film (Azqueta et al., 2013; Gutzkow et al., 2013). A commercial 'microarray' assay (CometChip) has also been developed (Watson et al., 2014). Scoring of comets in gels on the slides is time-consuming, and this presents a bottle neck in the performance of the assay, although semi-automated image analysis systems are available (Dusinska and Collins, 2008; Collins et al., 2022). Automated image analysis systems are also available (e.g., Metafer from Metasystems, Germany). To increase its sensitivity and to detect diverse types of lesions, the assay has been modified by the inclusion of a digestion with lesion-specific enzymes after the lysis step; thus otherwise undetectable base damage is converted into abasic sites and single strand breaks (SBs) are introduced (Dušinská and Collins, 1996; Olive, 2002). The most used enzymes are endonucleases specific for DNA base oxidation, namely formamidopyrimidine DNA glycosylase (Fpg) (Dušinská and Collins, 1996) or the mammalian counterpart, 8-oxoguanine DNA glycosylase (OGG1) which cleave oxidized purines, and endonuclease III (Endo III) for oxidized pyrimidines (Collins et al., 2014; Collins, 2017).

In this manuscript, we focus on application of the *in vitro* 12 mini-gel format alkaline version of the comet assay in combination with lesion-specific endonucleases (e.g., Fpg or

Endo III) for detection of both DNA SBs and oxidized DNA bases induced by NMs. We are addressing the most relevant points that need to be taken into consideration when assessing NM genotoxicity. Interpretation of NM comet assay data is facilitated by a categorization approach for positive, negative and equivocal effects recently developed within the H2020 NanoREG2 project (El Yamani et al., 2022).

A thorough physicochemical characterization of the NMs, both pristine as well as in culture medium should be always provided before toxicity testing. When performing genotoxicity, the cytotoxicity of the NMs to identify concentration range and the highest concentration must be conducted adequately. Last but not least, cellular uptake should be also investigated.

2. Materials and equipment

2.1 Materials

2.1.1 Consumables and reagents

Cells (adherent or suspension cells), Flasks 25 cm^2 or/and 75 cm^2, Glass microscopic slides, Cover slips 22 mm × 22 mm or 22 mm × 60 mm, Sterile plastic centrifuge tubes 15 ml and 50, Pasteur pipettes 2, 5 and 10 ml, 96-well plates, Microcentrifuge tubes (1.5, 5 ml), Serological pipettes, Pipette tips.

Cell culture medium (according to cell line) and additives (serum, penicillin-streptomycin, etc.), trypsin-EDTA solution (CAS. 59429C, Sigma), phosphate buffered saline (PBS) (Thermo Fisher, 10010049), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. number D5879- CAS. 67-68-5), Trypan Blue stain (Thermo Fisher, cat number 15250), Agarose—Electrophoresis grade normal melting point (NMP) (Fluka, cat number 05066), Agarose Low melting point (LMP) (Sigma-Aldrich, cat number A9414), distilled water, ethanol, Triton X-100 (Sigma-Aldrich, cat number T8787), Bovine serum albumin (BSA) (Sigma-Aldrich, cat number A9418), $CaCl_2$ (Mw = 74.55), $MgCl_2$, H_2O_2, 30%; (Sigma-Aldrich, cat number 31642-M), NaOH, Na_2EDTA (CAS 6381-92-6 SIGMA), Tris base (CAS 77-86-1 CALBICHEM), NaCl (CAS 7647-14-5 SIGMA), $KrBO_3$ (CAS 7758-01-2), KCl (CAS 7447-40-7 Sigma), HEPES (CAS7365-45-9 Sigma), KOH (Mw = 56.11), methymethane sulphonate (MMS) (CAS. M4016_ Sigma Aldrich), Fpg, Endo III, SYBR® Gold (Thermo Fisher S11494) (or other stains such as DAPI (4′,6-diamidino-2-phenylindole),PI (propidium idiode).

2.1.2 Equipment and software

Laminar flow hood, light microscope, countess cell counter or Bürker chamber with cover glass, pipettes, automatic pipettes and multi channel pipette (optional), microwave oven, CO_2 incubator, centrifuge, water bath or heat block, fridge 4°C, Incubator 37°C, electrophoresis equipment with power supplier, fluorescent microscope (with CCD camera).

For scoring comets, the use of a computer-assisted image analysis system with commercially available software is recommended to give the most reproducible results. Examples of scoring softwares: Comet assay IV (Instem), Comet Analysis software (Trevigen), Lucia Comet Assay™ software (Laboratory Imaging), Metafer (MetaSystems), KOMET 6 (Andor Technology). Several free scoring programs are also available such as Casplab or CometScore. The visual scoring system is an alternative (Dusinska and Collins, 2008) and (Collins et al., 2022).

2.1.3 Preparation of slides and solutions

2.1.3.1 Pre-coating of microscopic glass slides

Ordinary grease-free microscopic glass slides are pre-coated with (0.5%) normal melting point (NMP) agarose. To prepare 100 ml of agarose solution, weigh 0.5 g NMP agarose and dissolve in 99.5 ml distilled H_2O by heating in a microwave oven. Fill a suitable vessel (Coplin jar or a narrow beaker) with the hot NMP agarose solution and place it in a water bath or a heat block set at (55°C) for approximately 15 min before using it as described below step by step:

- Dip one clean microscope slide vertically in the solution of agarose by holding it from the frosted area.
- Drain off excess agarose by holding the slide vertically for some seconds, then wipe the back of the slide with a tissue and leave the slide horizontally on the bench to dry overnight.
- Mark the coated side with a pencil mark in one corner on the frosted end (e.g., top left) to identify the coated side.
- Dried pre-coated slides can be stacked together in slide boxes and stored at room temperature for several months.

Note. Commercially precoated slides are also available and can be purchased.

2.1.3.2 Preparation of low melting point agarose solution

The LMP agarose solution is made in PBS. The concentration can vary between 0.6 and 1% depending on the cell type and genome complexity. For instance, a lower percentage % of LMP agarose can be recommended when working with plants. For cultured cells, we recommend 0.8% LMP agarose. The agarose can be prepared in batches and stored at 4°C in a fridge. LMP agarose is dissolved in PBS by careful heating in a microwave oven; after about 10-15 s, shake the flask to ensure uniform heating; repeat until the fluid is clear and the agarose completely dissolved. Make small aliquots (e.g.,10 ml per bottle/falcon tube) and keep at 4°C.

2.1.3.3 Lysis solution

The preparation of lysis takes several hours to dissolve all reagents and to adjust pH. The lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, and 0.01 M Tris-base) is therefore usually prepared

ahead in distilled H_2O and kept at 4°C. Generally, all the ingredients are weighed and added before adding distilled H_2O. Triton X-100 at 1% is added to the lysis solution before use. The solution should be mixed properly using magnetic blender and kept at 4°C until use.

2.1.3.4 Enzyme reaction buffer for Fpg

The enzyme reaction buffer (0.04 M HEPES, 0.10 M KCl, 0.0005 M EDTA, 0.2 mg/ml BSA) is prepared in H_2O and the pH is adjusted to 8.0 using KOH (e.g., 8 M). The buffer is used to dilute the enzyme to the desired concentration. The buffer can be used for both Fpg and Endo III.

2.1.3.5 Electrophoresis solution

The electrophoresis solution (0.3 M NaOH, 0.001 M Na_2EDTA) is prepared in distilled H_2O and kept at 4°C.

2.1.3.6 TRIS-EDTA for dilution of SYBR®Gold

The TRIS-EDTA (TE) buffer (2.5 mM Tris-base, 4 mM Na_2EDTA) is prepared in distilled H_2O and pH adjusted to pH 7.5–7.8 (e.g., HCl). The buffer is also commercially available.

2.1.3.7 SYBR®Gold solution

To avoid repeated thawing and freezing, the commercially purchased SYBR®Gold stock can be aliquoted after first thawing (e.g., 50 µl in microtubes) and stored at −20°C. The SYBR®Gold may be further diluted in DMSO and stored at −20°C. On the day of staining of the slides, the stock dye is diluted 10 times in TE buffer. For the 12 mini-gels staining, a drop of diluted SYBR®Gold (20 µl) is placed on top of each gel. The slide is covered with coverslip 22 mm × 60 mm and placed in dark for 5–10 min before visualization under fluorescence microscope.

Be aware DNA dyes are carcinogenic and should be handled with care. Use gloves and collect the waste in a hazard-labelled container.

2.1.3.8 Enzyme preparation

The lesion specific enzymes used in combination with the comet assay are commercially available from different sources. The purchased enzymes are usually followed with instructions for their use. Here, we are describing the procedure for two lesion-specific enzymes used to detected oxidized bases, Fpg and Endo III. These enzymes are isolated from bacteria containing over-producing plasmids. Upon receipt, they should be dispensed into small aliquots (e.g., 5 µl) and stored at −80°C. The final dilution of the working solution varies from batch to batch. A titration of the enzyme is used to find the optimum dilution for comet experiments and is usually carried out by the supplier. The stock solution is diluted using the Fpg reaction buffer described above, with the addition of 10% glycerol; aliquots are stored at −80°C. For use in an experiment, the Fpg is thawed and further diluted with Fpg buffer (no glycerol is needed) following instructions from supplier. It is usually recommended to keep the aliquots all the time on ice until adding to the gels. If any of this working solution is left over, do not refreeze.

3 Methods

The standard alkaline comet assay procedure has been described in various papers, and in exhaustive detail in a recent Nature Protocols paper (Collins et al., 2022). Here we emphasize the particular considerations that need to be taken into account when applying it to NMs, but a brief outline of the overall procedure is in order. The principle of the assay is that strand breaks release the supercoiling in DNA loops and allow the DNA to extend towards the anode under electrophoresis, forming comet-like structures; the proportion of DNA in the tail represents the frequency of DNA breaks. A summary of the comet steps is presented in the Figure 1 below.

Cells that have been experimentally exposed to a NMs, accompanied by appropriate control cells, are mixed with LMP agarose and set as gels on a microscope slide (two large gels or 12 mini-gels) or on a GelBond film (up to 96 gels in a 12 × 8 array) or in more elaborated devices such as CometChip. The cells are lysed with high salt and detergent, leaving the DNA attached to the nuclear matrix as a so-called nucleoid. Digestion with lesion-specific endonuclease is an option at this stage. Electrophoresis follows, and the comets (typically 100 per sample) are quantitated using image analysis software or by visual scoring (Dusinska and Collins, 2008; Collins et al., 2022).

Due to its high sensitivity and to ensure reproducibility and reduce variability in the results, it is recommended to perform comet assay experiments always in the same manner following a standardized approach and experimental design taking into consideration, amount of medium to be used per treatment, plate layout type, dispersion of NMs and the series of controls (including agent control and reference standards) to be included (Dusinska et al., 2019). Moreover, historical data for negative (NC) and positive (PC) controls should be stored as they are key information for conclusion statement.

Additionally, to NC, capping agents' control, PC and interference controls, at least 4 concentrations of the test substance should be included.

The length of exposure to NMs is also crucial to consider as it should be sufficient for damage to occur. The comet assay normally measures an acute response and thus for testing chemicals *in vitro* an exposure time from 5 min (e.g., H_2O_2) to 24 h is usually recommended (Dusinska et al., 2019). However, for NMs testing we recommend at least three hours to ensure cellular uptake. An access to DNA could be dependent on dissolution of the nuclear membrane during mitosis (Catalán et al., 2014). Partly soluble NMs could exert their effects in shorter time. Generally, we advise both short (e.g., 3 h) and long (e.g., 24 h) exposure to be conducted within the same

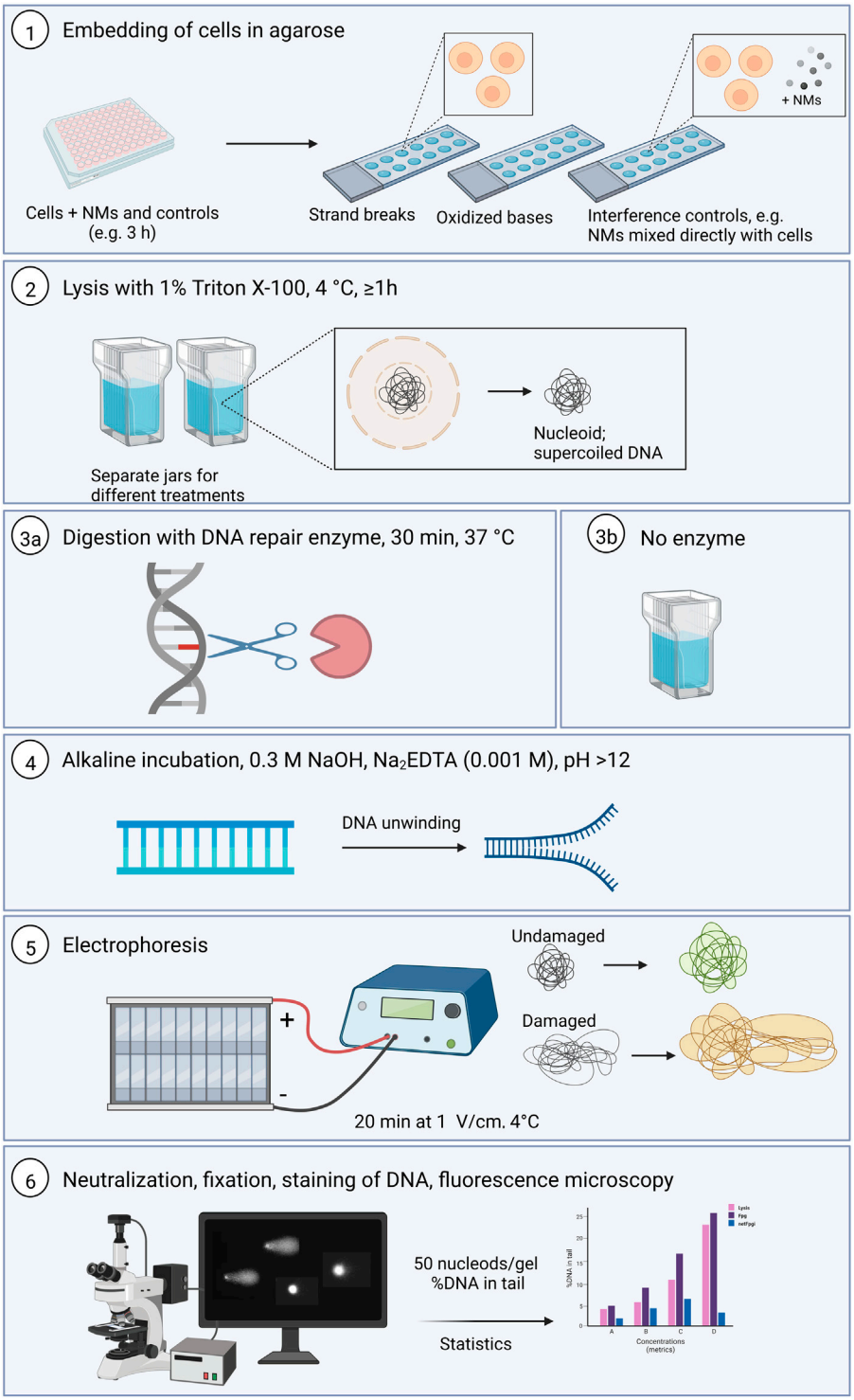


FIGURE 1
Summary of the comet assay protocol for both standard and the enzyme-modified version (Created with Biorender.com). 1. Cells are seeded in correct density using 96 well format and exposed to the NMs and controls and after exposure time, the cells are embedded with 0.8% LMP agarose to make 12-gel format slides. 2. Lysis incubation at 4°C for at least 1 h 3a. the slide with samples to be incubated with DNA repair enzyme to reveal oxidative damage are incubated with the enzyme for 30 min at 37°C. 3b. The slides with samples for DNA strand breaks detection remain in the lysis solution. 4. All the slides are placed in the alkaline solution for DNA unwinding. 5. Electrophoresis is run for 20 min at 1V/cm. 6. At the end of the unwinding, all the slides are washed by the neutralization solution, fixed and then stained before visualization and scoring. 50 nucleoids are analyzed per sample or gel. %DNA in tail parameter is collected and statistical analyses performed. h, hours; d, days; LMP, low melting point.

experiment. Three independent experiments (including at least two duplicates) are recommended.

When preparing the slides with 12 mini-gels and to increase the robustness of the results, it is recommended to include also replicate gels and replica slides in each experiment. Based on our experience, and due to the high sensitivity of this assay, it is also advised that the PC treatment should be placed in a separate plate or at least with empty wells separating them from the other samples. Also, when preparing gels on slides, the gels with cells treated with the PC should be made in separate slides.

3.1 Cell lines and preparation of culture

The comet assay has the advantage that it can be performed in both proliferating and non-proliferating cells. Any cell type with a nucleus can be used, and thus the assay can assess both cell- and tissue-specific DNA damage induced by NMs (Dusinska et al., 2019; Collins et al., 2022). For *in vitro* genotoxicity testing and for human hazard assessment of NMs, human and other mammalian cells such as from lung (e.g., A549 and Beas-2B cells), liver (e.g., HepG2 cells), circulatory system (e.g., THP1 or TK6 cells) are commonly used. The cells should be viable, and preferably at low passage (P). A guidance document was recently published about best practices in all aspects of the *in vitro* use of cells and tissues (Pamies et al., 2022). In this method paper, an example of adherent cells is given using the lung A549 cells. These cells (ECACC) grow in DMEM D6046 (low glucose with 4 mM L-glutamine) (Sigma), 9% fetal bovine serum (FBS) (26140-079, ThermoFisher), 100 U/ml penicillin/100 µg/ml streptomycin solution (15140-122, ThermoFisher). Suspension cells, such as human lymphoblastoid TK6 cells (ECACC) are grown in RPMI 1640 without glutamine (31870, GIBCO®, Life Technologies), 9% Horse Serum (16050122, GIBCO®, Life Technologies or H1138, Invitrogen), L-Glutamine 200 mM (25030-024, GIBCO®, Life Technologies), 100 U/ml penicillin/100 µg/ml streptomycin solution (15140-122, ThermoFisher). Cells are grown in complete culture medium and incubated in culture dishes or flasks in a cell incubator with humidified atmosphere at 37°C, 5% of CO_2 following the standard operating procedure (SOP) for cultivation of the specific cell line.

3.2 Seeding of cells for exposure

The seeding of cells can be conducted in any type of plate layout. However, to increase the throughput and the robustness of this assay, the use of 96 well plate format for cell seeding is recommended. The number of cells per well is dependent on the cell type and doubling time. For instance, for A549 cells, with a doubling time of about 22 h, it is recommended to seed cells 24 h before exposure to reach adequate confluency before exposure (70%–80%). A549 cells are normally seeded between 10.000–15.000 cells/well in 200 µl of medium in duplicate in a 96 well plate format. For TK6 cells, the seeding can be conducted on the same day or the day before exposure since the cells are in suspension. The cells are seeded at 15.000–20.000 cells/well in a 96 well plate in 200 µl final volume of medium.

3.3 Preparation of controls and nanoparticles

3.3.1 Negative controls

Concurrent NC handled in the same way as the treatment cultures should be included for every experimental condition as recommended by ENV/JM/MONO(2016) (OECD, 2017). The NC usually consists of cells incubated in the same culture medium for the specific cell line as exposed cells. It can also be the vehicle used such as PBS or DMSO. The vehicle controls should not produce toxic effects and should not be suspected to cause chemical reaction with the test substance.

PBS (with $CaCl_2$ and $MgCl_2$) may be used as NC but only for exposure times up to 2 h. If DMSO is used as a solvent for the NMs, it should be added to the culture medium or PBS in the same concentration as for the group exposed to the highest concentration of the test substance. The final concentration of DMSO should not exceed 2% (OECD, 2016).

3.3.2 Capping agent controls

The capping agent control(s) which are usually used to prepare the NMs are of utmost importance as stabilizers that inhibit the over-growth of nanoparticles and prevent their aggregation/coagulation in colloidal synthesis (Javed et al., 2020). The quality and the type of the capping agents are responsible for changing NMs physicochemical properties, and the biological characteristics affect they may have. Capping agents should be non-toxic and therefore, investigating their toxic potential separately is important along with testing the NMs suspension. Different types of capping agents have been used in nanoparticles' synthesis including surfactants, small ligands, polymers, dendrimers, cyclodextrins, and polysaccharides. The Polyethylene glycol (PEG), Polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), bovine serum albumin (BSA), ethylene diamine tetra acetic acid (EDTA) and chitosan are the most used capping agents for NMs. Capping agents can also be from plant extracts. Several studies have demonstrated the toxic effect of the NMs capping agents used when tested alone (Huk et al., 2015b). Information on the type of solvent, composition and concentration used need to be provided along with information about the NMs as pristine. The concentration of the capping agent to be tested has to be exactly the same in each cellular sample as in the vehicle control. Test at least a concentration of the capping agent used for the stock solution of the test substance equal to the amount in the highest concentration of the tested NM in the experiment. It is

TABLE 1 Examples of positive control chemicals to be used for the comet assay.

Substance name	Solvent	Diluted further in	Recommended stock concentration	Working concentration	Exposure time	Positive control for
MMS	DMSO + PBS	Cell culture medium	1 mM	0.1–0.3 mM	15–30 min, 1–24 h	DNA strand breaks
H_2O_2	—	Cell culture medium	100 mM	50–100 μM	5–30 min	DNA strand breaks
RO19-8022	70% ethanol + PBS	Cell culture medium	1 mM	1–2 μM	4–8 min	DNA oxidisd bases
$KBrO_3$	PBS	Cell culture medium	6 M	1–2 mM	3–24 h	DNA oxidisd bases

recommended to test also lower concentrations of the capping agents and to establish a concentration response curve.

3.3.3 Positive controls

Concurrent PCs should always be included, to demonstrate the ability of the method to detect a genotoxic effect under the conditions of the test protocol. If the treatment time for the PC is different from the exposure time for the tested NMs, the PC should be added towards the end of the exposure for the NMs so that NMs and PC exposures end at the same time. PCs can be selected according to the criteria of the specific study, the material tested, the method used and whether a metabolic activation system is present/needed. Some PCs may be used as reference standards that are applicable to several methods. In the case of the comet assay, MMS (alkylating agent) and H_2O_2 are commonly applied as reference standards and PCs for assay of DNA strand breaks. For DNA oxidized purines (Fpg-sensitive sites), potassium bromate ($KBrO_3$), or the photosensitizer Ro19-8022 in combination with visible light are used (see Table 1 for more information). When using H_2O_2, it is recommended to treat the cells after embedding for 5–10 min treatment (20–100 μM, 4°C) since with longer incubation H_2O_2 loses its activity and alsoDNA breaks are quickly repaired, (Collins et al., 2017a; Collins et al., 2022). The concentration of the PC to be used should be selected so as to produce moderate effects that critically assess the performance and sensitivity of the assay and could be based on concentration response curves established by the laboratory.

3.3.4 Preparation of nanomaterials, selection of concentration range and exposure

When testing NMs, proper dispersion of the material needs to be ensured. Information on dispersibility in terms of the relative amount of the NMs that can be dispersed in a suspending medium, including information on stability of the dispersion in the culture medium and the conditions applied should be provided (SCCS, 2019; EFSA et al., 2021). Depending on whether the material is in powder or suspension form, steps such as dispersion and sonication may be required. There is no universally applicable protocol for preparing stable dispersions of NMs, but specific methods for certain types of particles have been published, such as NANOGENOTOX protocol (NANOGENOTOX, 2012) for NM dispersion validated under several EU projects, namely FP7 NANoREG and H2020 NanoREG2 as well as H2020 PATROLS and RiskGONE. The EU-project NanoDefine has developed dispersion protocols for a number of NMs (Mech et al., 2020). The protocol developed by DeLoid et al. (2017) has also been applied by several EU projects, among them H2020 RiskGONE.

When exposing cells to NMs, two concentration metrics are normally considered, either mass per area (μg/cm^2) or mass per volume (μg/ml). The relationship between both metric units varies depending on the set-up (flask, dish, or multiwell). Other metrics include number of particles per ml or cm^2 as well as particle surface area per ml or cm^2. Whatever the concentration metric considered, it is important to provide all the information required to move from one metric to the others so that comparison of data will be facilitated.

The concentrations used for genotoxicity studies should be realistic and relevant to potential human exposure. The concentration range should be established with regard to expected cytotoxicity, solubility in the test system and changes in pH or osmolality (OECD, 2017). At higher concentrations, NMs have a tendency to sediment and to agglomerate, and therefore the highest concentration of NMs in tests should not exceed the level at which agglomeration is initiated (Catalán et al., 2014). The agglomeration of nanoparticles may affect their bioavailability to the cell and thus might lead to false positive/negative results. Within FP7 NanoTEST and NANoREG projects, it was agreed that the highest concentration should generally be less than 100 μg/ml. According to OECD TGs, the highest concentration should be below or up to the first concentration giving precipitation.

In general, at least four concentrations of the tested NMs should be included, plus negative/vehicle control (NC), PC and capping agents. For the comet assay, if the compound is cytotoxic, at least one cytotoxic concentration giving no more than 30% cytotoxicity, and a minimum of three non-cytotoxic concentrations, should be tested.

3.4 Nanomaterials primary and secondary characterization

Characterization of the NMs to be tested is a key to understanding their observed effects and their mechanism of

action. The physicochemical properties of NMs have been linked to their effect or toxicity in several studies (Huk et al., 2015b; Magdolenova et al., 2015; El Yamani et al., 2022). It is, therefore, important to perform full characterization of the pristine NMs where intrinsic properties will be measured; this is what we refer to as primary characterization. Behavior of NMs depends also on extrinsic properties which can be measured through so-called secondary characterization in the cell culture medium (size distribution, polydispersity, zeta potential, solubility, aggregation/agglomeration). There are several methods/techniques for NMs characterization, the most used being Dynamic Light Scattering (DLS) or NM tracking analysis (NTA) for size distribution and zeta-potential.

3.5 Cytotoxicity assessment as part of genotoxicity testing

It should be mentioned that a positive finding with the comet assay may not be due to genotoxicity but may also represent an indirect effect of general cellular toxicity. Therefore, cytotoxicity testing using an appropriate test should always be performed as part of all genotoxicity testing strategies. For the comet assay it is important to distinguish true exogenous DNA damage from the low level of DNA damage in the earliest stage of apoptosis. It has been recommended to limit testing to non-cytotoxic concentrations but a consensus about threshold has not been reached (Azqueta et al., 2022). There are several assays for cytotoxicity, some of which are time consuming when performing high throughput (HTP) analysis. The most used ones are based on colorimetric methods such as AlamarBlue, MTS, MTT, WST-1. However, potential interference of NMs with these methods needs to be tested (see Longhin et al., 2022 this collection of manuscripts). Cytotoxicity should always be tested with the same cells and the same set-up as for the comet assay—plate layout and amount of medium, NMs dispersion etc.—and ideally performed in the same experiment as the comet assay (El Yamani et al., 2017).

3.6 Cellular uptake and localisation of nanoparticles

It is now highly recommended to check internalization of NMs in the cells when testing genotoxicity. Accompanying genotoxicity testing with uptake studies is now required by several risk assessment committees (SCCS, 2019; EFSA et al., 2021). The aim is to demonstrate that cells are actually exposed, and that NMs are in contact with cellular organelles and molecules, including DNA. The DNA may be exposed to the NMs also during cell division, and so absence of nuclear uptake does not mean that NMs are not in contact with DNA. Demonstration of cellular uptake is particularly important when negative results are obtained. If such exposure cannot be demonstrated, a negative outcome of the assay might be meaningless, as the target exposure will not be known. However, a positive outcome from a genotoxicity test is not strictly dependent on uptake by the cells as genotoxicity may be induced *via* indirect mechanisms, such as through extracellular stimulation coupled to activation of intracellular signaling cascades, or *via* secondary genotoxicity by extracellular reactive oxygen and nitrogen species (Magdolenova et al., 2014; Dusinska et al., 2017a; Dusinska et al., 2017b). There are several methods to study uptake; most common are electron microscopy, confocal microscopy, Raman spectroscopy, flow cytometry and mass spectrometry (Dusinska et al., 2017b). Among them, transmission electron microscopy (TEM), is the most used (Huk et al., 2015b; Rubio et al., 2016; Kazimirova et al., 2019).

3.7 Comet assay procedure

On the day of exposure, the cells seeded in duplicate are exposed to the selected concentrations of the NM including positive(s), capping agents (s) and negative/vehicle controls and placed at 37°C, 5% CO_2, for the required time. Before the end of the exposure, the lysis solution is mixed with 1% Triton-X as described above. The final lysis solution is kept at 4°C until use. The LMP agarose is carefully heated in the microwave oven until completely melted and placed in a pre-warmed bath or thermo-block at 37°C until use. *Note*. Make sure the LMP agarose is at the right temperature 37°C before mixing it with the cells. Precoated slides should be labeled accordingly following a template.

3.7.1 Embedding of nanomaterials in low melting point agarose

At the end of exposure (day 1 or 2 depending on length of exposure), cells are mixed with LMP agarose. In the 12-gel fomat, each gel of 5–10 µl contains between 200–500 cells which is appropriate for image analysis. The volume of the cell suspension added to agarose to make the slides should not reduce the percentage of agarose to less than 0.45% (see also OECD TG 489 comet *in vivo*). The cell embeding should be done as soon as possible after cell treatment. From each treatment, 1- 2 gels are made on a pre-coated slide, preferably on 2 replicate slides. The slides for NMs interference control are prepared in parallel and as described above with one exception, no incubation time is needed for the NMs to be tested for interference. At the end of exposure, cells from NC and PC are kept to be used for the interference controls. The cells are mixed directly with the tested NMs in a way to achieve the highest tested NM concentration in the mixture. The mixture is then directly embedded in agarose as described above. Interference control slides are then placed into lysis solution and incubated into electrophoresis solution for DNA unwinding before electrophoresis, neutralization etc. as

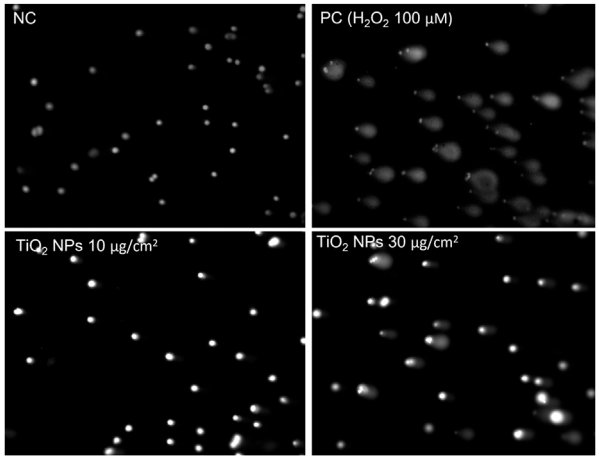


FIGURE 2
Example of analyzed samples with and without DNA damage. The nucleoids from the unexposed A549 cells (NC) were not affected with the electrophoresis and no increase in the tail was observed. While the nucleoids obtained from cells exposed to the PC (100 µM H_2O_2) and to TiO_2 NMs at 10 and 30 µg/cm² for 3 h show an increase in DNA migrated outside the nucleoids head forming a tail. The image analysis was performed after staining of the samples with SyberGold. For the image analysis Comet IV (Perceptive instruments) was used at ×100 magnification.

described above. The approach of using cells from the NC and then mixing directly with the NMs has been previously published (Magdolenova et al., 2012). There are also several other approaches to check for NM interference. For instance, for photosensitive NMs, such as TiO_2 NMs, comparison between results from performing the NMs exposure and embedding of cells on gels under normal light and red light or switching off the light has been suggested (Karlsson et al., 2015).

3.7.2 Immersion of slides in lysis solution

Lysis step is an important step and keeping constant lysis conditions will help avoid variability within experiments. Once prepared, the slides are immersed in cold lysis solution already prepared with 1% Triton-X and at 4°C and incubated for at least 1 h or overnight.

3.7.3 Unwinding in alkaline solution and electrophoresis

At the end of lysis incubation and enzyme incubation, the slides are then placed in the electrophoresis tank filled with cold alkaline electrophoresis solution, side by side, for the unwinding step. This step is also critical and the solution conditions, (e.g., pH, temperature), length of incubation and volume used should be kept constant. The slides (with gel drops) should be totally covered (0.5 cm of solution above). If there are gaps in the tank (few slides), it is recommended to fill the gaps on the platform with some empty slides to maintain the depth of solution over the platform. The period of incubation is usually 20 min at 4°C in dark. At the end of the unwinding step, the electrophoresis is conducted. Electrophoresis should be run at 4°C in a cold room or a fridge if possible. Within the hComet project, conducting electrophoresis at 1 V/cm for 20 min was recommended. The duration of electrophoresis is considered a critical variable and the electrophoresis time should be set to optimize the dynamic range. Longer electrophoresis times (e.g., 30 or 40 min to maximize sensitivity) usually lead to stronger positive responses with certain mutagens. It may also lead to excessive migration in control samples (Collins et al., 2022).

3.7.4 Neutralisation and fixation

After electrophoresis, slides are washd twice in cold PBS for 5 min follwoed by dH_2O for 5 min. The slide are left to dry horizontally at room temperature (normally overnight). Fixation using 70% ethanol for 15 min followed by absolute ethanol for another 15 min is recommended when using the 12-gel format. The slides are dried overnight and can be stored for months at room temperature as long as they are protected from light and dust.

3.7.5 Staining, image analysis and data collection

Before image analysis, the gels are stained with SYBR®Gold (0.1 µl/ml in TE buffer) or another specific dye such as DAPI (1 µg/ml DAPI solution in distilled H_2O). Slides are analysed using fluorescence microscopy with a computer image analysis program, e.g., Comet assay IV (Perceptive instruments), Metafer (Metasystems) or by visual scoring. We generally analyse at least 50 comets per gel (2 gels per treatment group). Cells close to the edge of the gel are not scored so as to avoid any potential "edge effects". It is recommended that every gel is scored "blind" to its treatment. This is the standard practice for studies conducted in a regulatory environment, for example under Good Laboratory Practice (Bright et al., 2011). The % DNA in tail is considered the most informative parameter (Collins, 2004; Møller et al., 2014; Møller et al., 2020; Collins et al., 2022).

An example of results from non treated A549 cells (NC) and cells treated with TiO_2 nanoparticles are shown in Figure 2.

At the end of image analysis, the data are collected in suitable templates. To facilitate data collection and interpretation, the use of harmonized templates to report comet data is highly recommended. Within several EU projects, data collection template has been introduced for the comet assay, i.e., by the FP7 NanoTEST and NANoREG projects. The template has been further improved within H2020 RiskGONE to move towards data reporting, harmonization, and data FAIRness. The template provides a function for automatic calculation of the results from the reported raw data. The template is available upon request through the eNanomapper database (www.enanomapper.com), and it will be made publicly available. In Figure 1, we summarized the main steps of this assay.

4 Data analysis and statistics

The use of an appropriate statistical programme is recommended (e.g., Excel, GraphPad Prism, SPSS). In general, data from comet assay are processed as follows:

- Calculate the median of the ±50 comets (% DNA in tail) per gel replicate.
- Calculate the mean of medians and standard deviation SD (for the replicate gels of the same concentration/sample within the same experiment). Then, calculate the mean value ±SD for all independent experiments (at least three independent experiments are recommended).
- Compare the DNA damage of the PC with NC (control group),
- Compare the DNA damage of the tested NMs with NCcontrol group). Consider differences between replicates, differences between controls and treated cells, correlations and concentration response relationship.

The choice of the statistical tests to be applied, parametric or non-parametric tests, depends on many factors such as size of the data, data distribution, number of repeats. For more information see (Lovell and Omori, 2008; Bright et al., 2011; Lovell, 2012).

5 Test acceptance criteria

For an experiment to be considered valid, it needs to include:

- Valid PC: The PC used in the comet experiments is valid or acceptable, if the effect is within the range of mean ±2× standard deviation of historical control data for the same cell line.
- Valid NC: The NC is valid if the effect observed is within the range of mean ±2× standard deviation of historical control data for the same cell line.
- Adequate number of cells and concentrations have been analysed.
- The criteria for selection of the highest concentration of the NMs are met.
- Quality control of test system (mycoplasma test) is shown to be negative

6 Historical positive and negative controls

For every laboratory using *in vitro* comet assay, it is highly recommended to build historic controls, both negative as well as positive, for each cell type used. Different cell lines may give different % DNA in tail (background damage level) for the NC. It is also important to demonstrate the ability of the laboratory to perform the assay consistently and to show that the cells used have a low background level of DNA damage, so are capable of picking up a positive effect, with reasonably low variability (OECD, 2014b). When reporting results, it is informative to show average and minimum-maximum values of negative/positive historical controls from last 10–20 experiments performed in the laboratory.

A laboratory´s historic database for NC and PC data for relevant cell lines needs to be up to date. To define the acceptable range for DNA damage level on NC and PC, controls, calculated Mean ±SD of the data can be used. With the aim of monitoring the proficiency of the *in vitro* assays, both initially and over time, the use of quality control charts to assess the historic control databases is recommended (For more information, see report on statistical issues related to OECD TGs on genotoxicityand Genetic toxicology Guidance documents) (OECD, 2014a, 2015). An example of historical controls from the NC using A549 cells without enzyme treatment from our laboratory is presented in Figure 3.

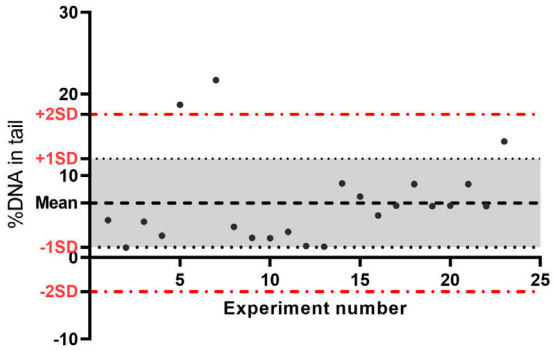


FIGURE 3
Data quality control chart of negative controls (no enzyme) from 22 experiments performed over time on A549 cells. The central line represents the average of all negative control data, the upper line (red) is for the upper control limit (+2SD), and the lower line (red) is for the lower control limit (–2 SD). The lines are determined from the laboratory historic data. By comparing current data to these lines, we can draw conclusions about whether the negative control is acceptable; if the value is outside the upper line, the negative control is not acceptable. SD, standard deviation.

7 Evaluation and interpretation of the results

In addition to fulfilment of the acceptance criteria, since in the case of NMs a concentration response is not always observed (due to agglomeration at higher concentrations), in the EU FP7 project NanoREG2 we developed modified criteria for

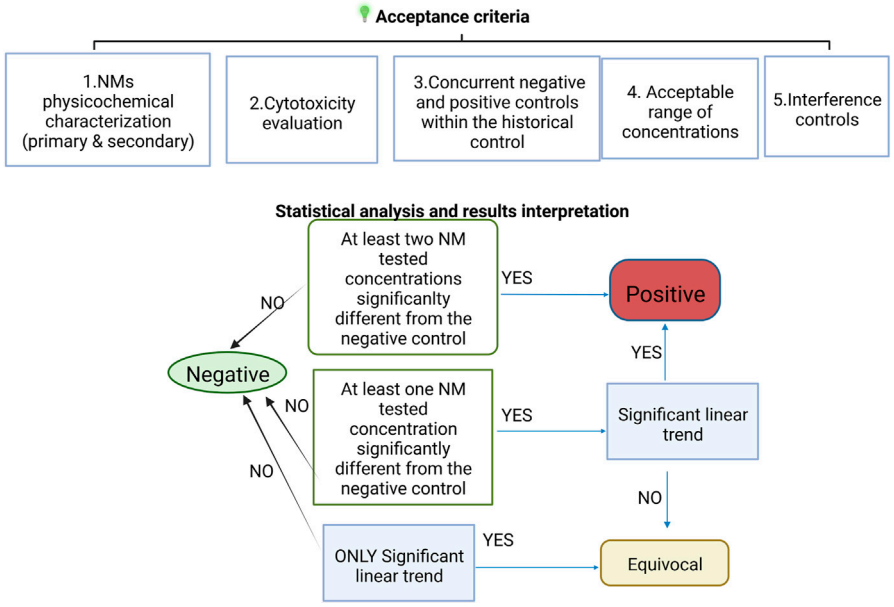


FIGURE 4
Scheme of the test acceptance criteria for NMs testing including the interpretation of the statistical analysis results.

positive, negative and equivocal genotoxicity response (El Yamani et al., 2022).

A compound is considered positive if there is:

A. a significant increase in strand breaks or oxidised DNA bases at two of the tested concentrations (<30% cytotoxicity) compared to negative controls OR
B. a significant increase in strand breaks or oxidised DNA bases at one of the tested concentrations compared to negative controls AND a concentration response relationship when evaluated with an appropriate trend test.

A compound is considered equivocal if there is a significant concentration response OR a statistically significant increase in strand breaks or oxidised DNA bases at one of the tested concentrations (<30% cytotoxicity) compared to negative controls.

A compound is considered negative if none of the above criteria are met; additionally, all results are inside the distribution of the historical negative controls.

A scheme summarising the acceptance criteria and evaluation of the NMs effect is shown in Figure 4.

To summarize, negative results indicate that, under the test conditions, the tested NMs does not induce DNA damage in the cultured cells used. Positive results indicate that, under the test conditions, the NM tested is potentially genotoxic *in vitro*. If the response is neither clearly negative nor positive, the test substance is considered equivocal and further testing is needed.

8 Interpretation of interference control results

The interpretation of results from interference controls depends on the set-up followed. For instance, if the controls are intended to investigate whether a direct physical interference may occur after cell embedding between residual NMs and DNA, influencing DNA migration, results are compared with those from the controls without NMs. If there is a significant increase or decrease in % DNA in tail compared with the NC or PC, we may conclude that there is an interference. More details on how to perform and interpret interference controls are under preparation for a separate manuscript.

9 Discussion

The miniaturized enzyme-linked comet assay is one of the few key assays available to study DNA damage and DNA oxidation induced by NMs. In this paper we described an optimized version of this assay *in vitro* which has been applied in several projects including EU-projects NanoTEST, NanoTOES, NANoReg, NanoREG2 (Magdolenova et al., 2014; Garcia-Rodriguez et al., 2019) and others; it is also being followed within the current projects H2020 RiskGONE and NanoSolveIT, to carry out hazard assessment of several NMs.

The miniaturized version of the comet assay is enormously advantageous for NMs testing, enabling testing of a large number

of NMs within the same experiment, minimizing variability and increasing robustness. The miniaturization of the comet assay using 12- gel or even 96 gel format has already been successfully applied to study many NMs (Azqueta and Dusinska, 2015; Di Bucchianico et al., 2017; El Yamani et al., 2017; Garcia-Rodriguez et al., 2019; Collins et al., 2022).

Owing to their physicochemical properties, NMs are more challenging to test for genotoxicity than their counterpart bulk chemicals. The conventional procedure for *in vitro* comet assay testing of chemicals has been adapted to meet the specific needs of NMs testing. Acceptance criteria have been revised, as well as requirements for test validity. The effect of NMs on DNA is highly related to their physicochemical properties both intrinsic (as pristine) and extrinsic (e.g., in medium, vehicle). Characterization of NMs in terms of pristine TEM size, size distribution in cell culture medium before during and after exposure, dissolution rate, zeta potential, and cellular uptake is needed to fully understand the mechanisms and mode of action of NMs (Huk et al., 2015a; Magdolenova et al., 2015). When assessing NMs for their hazards, it is necessary to follow a standard protocol of dispersion and sonication, even including calibration of the sonicator. We previously published a testing strategy to increase the robustness and the throughput of this assay, allowing testing several NMs, different cell lines, different time points and different endpoints within same experiment (Dusinska et al., 2015).

Interpretation of comet assay data when testing NMs may also be challenging. The concentration-response relationship for NMs is not straightforward as it is for chemicals; in other words, an increase in concentration does not necessarily mean an increase in effect. On the contrary, it may lead to an increase of aggregation/agglomeration which will affect the cellular uptake and final effect. The comet assay is a sensitive method and the background level of DNA damage in cells varies. Historical controls are important to demonstrate the technical competence of a given laboratory, and its familiarity with the assay (Hayashi et al., 2011). The OECD has clearly stated how important it is to compare control data in a given experiment with historical controls (negative and positive) for the cell lines to be used (OECD, 2015). The interpretation of genotoxicity results was discussed at the 2009 International Workshop on Genotoxicity Testing (IWGT) in Basel, Switzerland (Hayashi et al., 2011). We stress the importance of historical controls data and encourage users of this assay to build their historical control database as recommended by the OECD (Hayashi et al., 1989; Adler et al., 1998). Providing historical control data, or other proof of validity of the assay, should be a requirement when publishing *in vitro* comet assay data relating to the hazard assessment of any substance, including NMs.

The selection of concentrations of an NMs for genotoxicity testing *in vitro* can only be defined when information on cytotoxicity is available. Exposure to cytotoxic concentrations can lead to false positive genotoxicity results, and so definition of the cut-off for cytotoxicity is important. We also recommend the use of modified criteria for positive, negative and equivocal response for NMs. The responses can be expressed by numbering each category—1-negative, 2-equivocal and 3-positive. This is relevant when integrating physicochemical properties and *in vitro* toxicological data with *in silico* tools as described in El Yamani et al., 2022 and for developing predictive models.

Potential interference of NMs with the testing methods has become a topic of concern already for many years. Most conventional toxicity assays rely on colorimetric/fluorometric principals, and the particular physical properties of NMs mean that they are prone to interfere with testing methods, as shown in several studies (Ong et al., 2014). Possible interference between NMs and the comet assay has been investigated (Kain et al., 2012; Magdolenova et al., 2012; Karlsson et al., 2015; Ferraro et al., 2016; George et al., 2017; Jalili et al., 2022). For instance, Ferraro et al., 2016, showed a possible interaction between naked DNA and NMs just before the electrophoresis step (Ferraro et al., 2016). Other authors questioned the use of comet assay for testing photosensitive NMs (Karlsson et al., 2015). Therefore, we strongly stress the importance of including additional controls to check for possible NMs interference. A thorough approach is being developed further under H2020 RiskGONE; a joint review paper on NMs interference is under preparation.

The comet assay is widely used for testing genotoxicity of chemicals and is the most used method for testing genotoxicity of NMs. It is thus important to follow standard protocol that addresses all challenges related to NMs features. Hence, to develop OECD TG for *in vitro* comet assay is urgently needed.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

NE wrote the manuscript. NE, MD adapted the SOP for the NMs testing. MD, AC supported the writing and the English. All authors reviewed the manuscript.

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Conflict of interest

Author ARC was employed by Comet Biotech AS.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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