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ADVANCED REVIEW

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Genotoxicity testing of nanomaterials

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Abstract

Nanomaterials have outstanding and unprecedented advantageous material properties but may also cause adverse effects in humans upon exposure. Testing nanomaterials for genotoxic properties is challenging because traditional testing methods were designed for small, soluble molecules and may not be easily applicable without modifications. This review critically examines available genotoxicity tests for use with nanomaterials, including DNA damage tests such as the comet assay, gene mutation tests such as the mouse lymphoma and *hprt* assay, and chromosome mutation tests such as the micronucleus test and the chromosome aberration test. It presents arguments for the relative usefulness of various tests, such as preferring the micronucleus test over the chromosome aberration test for scoring chromosome mutations and preferring mammalian cell gene mutation tests because the Ames test has limited utility. Finally, it points out the open questions and further needs in adapting genotoxicity tests for nanomaterials, such as validation, reference nanomaterials, and the selection of top test concentrations, as well as the relevance and applicability of test systems and the need to define testing strategies.

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DNA damage, genotoxicity, mutagenicity, nanomaterials, particles, test methods

1 | INTRODUCTION

Nanomaterials (NMs) are variably defined, most commonly as materials possessing a size of ≤100 nm in at least one dimension. One consequence of this exceedingly small size is a very high surface-to-volume ratio, which leads to an unusually high reactivity of everything located on the surface of the respective NM. This allows for unprecedented desirable possibilities but also leads to the potential for toxicities involving high activities and possibly even new

qualities. Because of the novel properties of NMs, the traditional forms of toxicity tests are not optimal in every case for the investigation of these novel materials.

Genotoxicity, which is responsible for damage to the genetic material DNA, is especially important since it leads in many instances to profound disturbances, including cancer.

Genotoxicity investigations on NM have been reviewed by us (Landsiedel et al., 2009; Oesch & Landsiedel, 2012) and by others (e.g., Charles et al., 2018; Dusińska et al., 2019; Gonzalez et al. 2008, 2011; Kohl et al., 2020). The aim of this review is a systematic presentation of the genotoxicity tests used for NMs, including indications of shortcomings and thoughts on possible optimizations to enhance their use for NMs.

2 | GENERALITIES CONCERNING MANY OR ALL PRESENTED TESTS

2.1 | Mechanisms of NM-dependent genotoxicity

NMs can lead to genotoxic effects by different mechanisms, whose discrimination is important for risk assessment:

- primary mechanisms due to direct interaction of the NMs with DNA (primary direct mechanisms),
- primary indirect mechanisms (interaction of NMs with molecules involved in DNA replication, transcription and repair as well as in cell division) and
- secondary mechanisms, such as NM-dependent inflammation leading to the generation of ROS, which in turn attack DNA.

As discussed in this review, it appears that most genotoxic damage produced by NMs is due to secondary mechanisms, such as damage to cellular organelles, including lysosomes and mitochondria, leading to the leaching of ROS and other reactive species as well as inflammation-dependent oxidative stress, finally producing oxidative DNA lesions. These secondary mechanisms require finite threshold doses to produce the initial lesion, for example, inflammation. However, in some cases, clear evidence was obtained for primary genotoxicity evoked by certain NMs, such as unambiguously inflammation-independent DNA damage (e.g., as ascertained by the comet assay after pulmonary instillation of low doses of carbon black NPs [Printex 90, primary particle size 14 nm] in mice; Kyjovska et al., 2015).

A major advantage of in vivo NM genotoxicity tests is the possibility of recognizing secondary genotoxicity dependent on NM-provoked inflammation leading to ROS-dependent DNA damage. Important factors in such tests include the following: (1) Doses must be relevant. Secondary genotoxicity occurring only at high doses may not be relevant in real life. (2) Secondary genotoxicity must be fundamentally differently evaluated, as it possesses a threshold dose.

Since in vivo tests are for ethical reasons usually performed in animal models, the interpretability of the results obtained from them is obviously limited by substantial interspecies differences.

2.2 | Battery of tests

No strict protocol exists for investigating the potential genotoxicity of NMs by using a battery of tests, yet it is obvious that NM-induced genotoxicity can involve different mechanisms (see above), necessitating the use of multiple assays. Regarding in vitro tests, a very sensitive test for early DNA damage, such as the comet assay, may—outside a recommended battery—be taken as an initial pretest, although the comet test suffers from rather low specificity, and the DNA damage recognized in such tests will not necessarily be transformed into stable mutations. A gene mutation test (preferably in mammalian cells, such as the MLA [OECD TG 490; 2016g] or the HPRT [OECD TG 476, 2016d] test) combined with a test for chromosome mutations (such as the CBMN [OECD TG 487, 2016e] or the CA test [OECD TG 473, 2016a]) may be recommended as members of a battery of in vitro NM genotoxicity tests.

In vivo tests appear to be necessary only if in vitro tests give positive results or are not suitable for the given problem. In vivo tests that can be recommended for investigating the potential genotoxicity of NMs include an in vivo mammalian alkaline comet assay (OECD TG 489, 2016f), an in vivo micronucleus test (OECD TG 474, 2016b) (alternatively an in vivo CA test [OECD TG 475, 2016c]), and/or a transgenic rodent somatic and germ cell mutation assay (OECD TG 488). It is, however, worth mentioning that the assessment of target organ exposure is an essential part of the

interpretation of genotoxicity results. In many cases, multiple treatments might be necessary to facilitate exposure of the target organ.

2.3 | Cell cultures

The response to genotoxic insult by NMs can vary enormously among cell lines. Major variables contributing to these differences in response include differences in uptake of the tested NMs (Elespuru et al., 2018), differences in DNA repair and in antioxidant equipment (Hanot-Roy et al., 2016), and overall differences in genetic background (Li, Doak, et al., 2017). As an example: A positive response to TiO_2 NM (anatase:rutile 79:21) in the Fpg Comet assay using alveolar A549 cells but a negative response in bronchial BEAS-2B cells was reported by Ursini et al. (2014). Possible reasons for this day and night difference include a stronger response to oxidative stress in A549 cells due to a mutation in the KEAP1 gene (Biola-Clier et al., 2017) or the production of pulmonary surfactant in A549 cells, which can change the surface properties of the NM (Armand et al., 2016). However, Biola-Clier et al. (2017) observed no difference between these two cell lines, A549 and BEAS-2B cells, in response to TiO_2 NM (NM105 TiO_2, 21 ± 9 nm, anatase:rutile 86:14). A549 cells have been criticized for an abnormal response to oxidative stress (Singh et al., 2006). On the other hand, A549 cells subjected to acute TiO_2 exposure showed a genotoxicity response similar to that of another human cell line, BEAS-2B (Biola-Clier et al., 2017). On the grounds of the enormous differences between cell lines in visualizing the sensitivity to genotoxicity, it is especially important to ascertain the adequate performance of a cell line for testing NMs at least by the use of appropriate positive and negative controls in the test. Although high sensitivity is highly appreciated in a test system, the results should be confirmed in relevant in vivo follow-up studies.

2.4 | Higher-complexity models: 3D cultures and cocultures

EpiAirway™ 3D human bronchial models proved useful for investigating NMs under conditions similar to physiological conditions in humans. In the study by Haase et al. (2017), most of the 16 investigated NMs were not genotoxic (surface-modified 15 nm SiO_2 [3 variants], 10 nm ZrO_2 [3 variants], nanosilver [2 variants], TiO_2 NM-105, $BaSO_4$ NM-220, 2 AlOOH NMs). Genotoxicity was observed only for ZnO and Ag.50.citrate and marginally for unmodified SiO_2, SiO_2 phosphate, and ZrO_2.TODS. Genotoxicity was tested by the standard alkaline comet assay. Interestingly, 50 nm nanosilver with citrate functionalization showed positive results, while the PVP-coated 50 and 200 nm nanosilver showed negative results. As expected, the results obtained in the 3D model agreed well with the in vivo results and were much better than the results obtained in standard 2D cell cultures. One major reason for the better agreement of the 3D model with in vivo data appears to be a much lower NM uptake rate in both situations (3D model and in vivo) compared with 2D models. In the 3D model, the uptake was below 5% of the applied dose for all NMs for which uptake could be measured (tested by inductively coupled plasma–mass spectrometry [ICP–MS]). In standard 2D cell cultures, uptake of up to 12% (Gliga et al., 2014) or even 50%–60% (Kettler et al., 2016) of the applied dose has been observed in much shorter time periods (24 h) than in the presented 3D study (60 h) (Haase et al., 2017).

Prasad et al. (2020) also concluded that 3D cell cultures are more resistant to the genotoxicity (as well as cytotoxicity and morphological disturbances) produced by NMs (ZnO NPs), in line with an architecture more closely related to the in vivo situation, which further agreed with findings by Chen et al. (2019) on Ag NPs. Fleddermann et al. (2019) observed that SiO_2 NPs entered a HepG2 spheroid only to a depth of 20 μm during an exposure of 24 h, but the NM was present throughout the spheroid if it was mixed with the cells prior to spheroid formation.

Several studies using micronucleus testing as a genotoxicity endpoint also concluded that 3D cultures were more resistant to NM-induced genotoxicity than 2D cultures. Wills et al. (2016) observed no induction of MN upon the exposure of 3D EpiDerm™ tissue to Levasil^R silica NPs of 16 and 85 nm, while in the same experiment, the 2D culture of TK6 human B lymphoblastoid cells showed a clear increase in MN. The authors noted that the 3D skin model that they used, although clearly closer to the in vivo situation than the 2D culture, may still not fully mimic the in vivo situation with respect to potential wound- or follicle-facilitated exposure. Moreover, since differentiated cells are used for 3D cultures, they replicate relatively slowly. Hence, it is critical to ascertain that the replication rate is sufficient for a meaningful MN test (Haase et al., 2017). Additionally, it should be ascertained that the test article has penetrated to the replicating cells, and the exposure times may have to be longer than for cell lines (i.e., 48–72 h for 3D cultures) (Dusińska et al., 2019). Repeated dosing (e.g., 0, 24, and 48 h) may be advisable (Haase et al., 2017).

Very interesting 3D models supporting longer-term exposure durations have been developed by the Doak group (Conway et al., 2020).

Coculture of more than one cell line can improve the simulation of in vivo situations. Dorier et al. (2019) used a coculture of 70% Caco-2 cells together with 30% HT29-MTX cells as a model for the intestine to investigate the potential genotoxicity of TiO_2 NMs in the form of the food additive E171 (primary particles 118 ± 53 nm) as well as NM105 (primary particles 24 ± 6 nm). TiO_2 NMs were dispersed by high-energy (47.7 W) sonication in water (30 min, 4°C), leading to aggregates with a hydrodynamic diameter of 415 ± 69 nm for E171 and 158 ± 1 nm for NM105. For cell exposure, they were then diluted in complete cell culture medium, in the presence of fetal bovine serum (FBS). Caco-2 cells were proliferative (not differentiated), while HT29-MTX were already producing mucus. These conditions were chosen to account for physiological parameters: intestinal epithelial cells exhibit a differentiation gradient from the bottom of crypts to the top of villi and are constantly covered by mucus. The presence of FBS, leading to a protein corona on the tested NM, mimics the situation of ingested food being covered by salivary and intestinal proteins before entering the intestine. Interestingly, the authors found an increase in intracellular ROS but no oxidative DNA damage using the alkaline comet assay in the presence of Fpg (nor any impairment of cell viability). The TiO_2 concentration was up to 200 μg/ml, which is a high concentration compared with the estimated human daily intake (Weir et al., 2012). These negative findings agreed with many (Dorier et al., 2015; McCracken et al., 2016; Shi et al., 2013; Skocaj et al., 2011; Song et al., 2015) but not all (Gerloff et al., 2009; Proquin et al., 2017a, 2017b) previous studies on TiO_2 NMs. The authors discuss possible reasons for the discrepancies between their studies and the latter two, which may be worthwhile to present briefly here for the benefit of the readers. Dorier et al. (2019) dispersed TiO_2 particles by sonication in water and then immediately diluted them in cell culture medium containing FBS since steric repulsion by serum proteins hinders the formation of large agglomerates. Proquin et al. (2017a), however, dispersed TiO_2 particles by sonication in cell culture medium containing bovine serum albumin (BSA). Sonication of protein leads to denaturation and aggregation (Stathopulos et al., 2004). Dorier et al. (2019) used high-energy sonication, leading to stable suspensions with an average hydrodynamic diameter of 739 ± 355 nm of E171 and 440 ± 7 nm of the NM105 particles, while Proquin et al. (2017a), using bath sonication, obtained large agglomerates of >1000 nm, which sediment rapidly, thereby increasing exposure of the cells in line with the positive genotoxicity outcome in their study (also see the nanogentox protocol for the formulation of NM and the relationship of sonication with deagglomeration: Alstrup Jensen et al., 2014). Moreover, Dorier et al. (2019) used a coculture including HT29-MTX cells secreting mucus, which protects cells from NMs, similar to the situation in the intestine described above, while Proquin et al. (2017a) used Caco-2 cells as a monoculture. Similar conditions held for the positive outcome of the study by Gerloff et al. (2009). They used a monoculture of nondifferentiated Caco-2 cells and exposure of the cells in serum-free medium. On the other hand, it should be noted that the coculture used by Dorier et al. (2019) contains no immune cells, the main drivers of inflammatory response and ROS generation, which can lead to secondary genotoxicity. A 3D model containing intestinal epithelial cells as well as immune cells (Susewind et al., 2016) may represent an option to recognize secondary genotoxicity, and the use of both systems may allow us to discriminate primary from secondary genotoxicity, keeping in mind that secondary genotoxicity may rightfully be assumed to be thresholded, while direct primary genotoxicity is assumed to not to be thresholded normally. Evans et al. (2019) developed (a) a conditioned-medium-based system of treating differentiated dTHP-1 macrophages with gamma-Fe_2O_3 or with Fe_3O_4 superparamagnetic iron oxide NPs (SPION) and then transferring the medium to 16HBE14o$^-$ bronchial epithelial cells; (b) a coculture consisting of differentiated dTHP-1 macrophages and 16HBE14o$^-$ bronchial cells treated with SPION. They compared these systems with a monoculture of 16HBE14o$^-$ bronchial cells treated with either of the two agents. Potential genotoxicity was investigated using the CBMN test. Positive results for SPION were obtained only in the conditioned medium culture and in the coculture but not in the monoculture, although the cellular uptake of SPION was demonstrated by electron microscopy for all three systems. The authors conclude that their novel systems, and likewise quite generally multi-cell-type models, will help to visualize a wider breath of potential genotoxic damage, in their case immune-reaction-dependent secondary genotoxicity. Coculture has an additional advantage over the conditioned-medium approach in that it allows direct contact between different cell types.

The use of 3D cultures and cocultures can also help to simulate the in vivo situation more closely. For example, Fukai et al. (2018) showed that the mutation spectrum produced by multiwalled carbon nanotubes in a coculture of murine lung resident cells (GDL1) and immune cells (RAW264.7) was very similar to that produced in vivo but very dissimilar from that produced in the GDL1 monoculture.

2.5 | "Close to human physiology" test conditions

It is obvious that the use of conditions mimicking those in the human organism is preferable, such as using human cells, human serum, and medium providing a chemical composition similar to that of human plasma. It should, however, be confirmed that there is no interference with the scope of a test on the potential genotoxicity of NMs.

2.6 | Cytotoxicity

The highest concentrations of NM in genotoxicity tests should be oriented either at the presumptive human exposure or at the cytotoxicity that the tested NM exerts in the test organism used. Excessive cytotoxicity can lead to false positives in genotoxicity testing. During cell death, double-strand DNA breaks occur without being due to primary or secondary genotoxicity. It is therefore important to follow the guidelines concerning cytotoxicity, such as those set by the OECD, which are quoted further below in the discussions of the individual tests. Additionally, the preferential use of individual cytotoxicity tests for individual genotoxicity tests of NMs is discussed further below under the individual tests, where appropriate.

2.7 | Characterization of NMs

It is probably commonly understood by now that NMs of the same chemical composition can occur with different characteristics, leading to fundamentally different testing results. It is therefore important to determine these characteristics and report them together with the results of the genotoxicity tests (cf. Krug, 2018). These characteristics include the NM size, surface area, shape (spheres, plates, tubes, aspect ratio), crystalline structure, surface roughness, charge (zeta-potential), coating, formation of a protein corona (potentially promoting endocytosis or masking reactive sites), dispersion, dissolution rate, aggregation/agglomeration, and stability of the respective NM in the test medium (at the very least, the size distribution, dissolution, and surface area [BET] in the medium should be analyzed).

Methods for determining size and shape include dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA); for the determination of shape, size, size distribution and agglomeration, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM); for the determination of surface charge, the measurement of the zeta potential; for the separation of different particle sizes, field flow fractionation combined with UV, and multiangle light scattering (MALS), which, if additionally combined with inductively coupled plasma–mass spectrometry (ICPMS), allows the determination of the composition and concentration of the respective solutions/suspensions (for details and references see Kohl et al., 2020). The presence versus absence and the quality of a coating need to be carefully characterized, since coatings can profoundly influence and even eliminate genotoxicity (as shown, e.g., by Lacerda et al., 2008 for multiwalled carbon nanotubes coated with diethylene triamine pentaacetic dianhydride).

The primary particle size and size of aggregates/agglomerates are of high importance, yet in a somewhat complicated, nonuniversal and nonunidirectional way (Dekkers et al., 2018 and references quoted therein). Maser et al. (2015), using an alkaline unwinding assay, observed DNA strand breaks in V79 cells exposed to 15 nm but not to 55 nm amorphous SiO_2 NMs, which was consistent with many studies that show higher genotoxicity (as well as other biological activities) of smaller-sized NMs of the same chemical composition, as generally expected from their higher active surface-to-volume ratio. However, in some experiments, higher genotoxicity was observed for larger-sized NMs of the same chemical composition. The differences in biological activities between NMs of different primary and/or aggregate sizes appear to become substantially smaller if the dose is expressed in terms of surface area (mm^2 NP/cm^3 tissue) instead of mass (μg NP/g tissue) (Dekkers et al., 2018; Oberdörster et al., 2005). With respect to agglomeration size, Llewellyn et al. (2021) showed, in a highly physiological 3D model of the human liver, that the top dose (10 μg/ml) for all five investigated NMs (TiO_2, ZnO, Ag, $BaSO_4$, and CeO_2) led to a lower genotoxicity (as determined by the CBMN assay) than the second highest dose (5 μg/ml), in line with the increasing agglomeration sizes with increasing doses determined within the same study. This clear-cut inverse level of genotoxicity with respect to concentration is likely due to more restricted translocation of the larger aggregates through the compact spheroid, mimicking physiological conditions in living tissues.

The actual size of NMs as well as other properties should be investigated under the conditions of the test, ideally at the beginning and end of the test and, in addition, ideally taking into consideration the intended human exposure. This is of fundamental importance with respect to possible agglomeration in the test milieu as well as the possible corona formation of adsorbed proteins and other molecules of the medium on the surface of the tested NM. When NMs come into contact with cell culture medium, their surface properties change, as do their particle–cell and particle–particle interactions (Precupas et al., 2020), depending on the properties of the primary particle and the milieu (Moreno-Villanueva et al., 2011). Moreover, the aggregate size increases with increasing NM concentration, and the increase in aggregate dispersal aid by increasing serum concentration decreases at higher NM concentrations (Doak et al., 2009). The rather complicated, nonuniform dependence of the outcome of genotoxicity tests on NM in cells in culture on the presence and concentration of serum in the medium may lead to the recommendation to routinely use more than one concentration of serum as well as NM aggregate size measurement at more than one concentration of the tested NM.

2.8 | Characterization of the test milieu

The medium in which the genotoxicity test on NM is performed profoundly influences the outcome of the test and therefore needs to be carefully reported. This includes its composition, such as the presence/absence/concentration of serum and macromolecules (such as proteins and peptides) and the pH, ionic strength, ion valence, hydrophobicity, and viscosity of the medium.

2.9 | Photoactivation

An important aspect for the appropriate use of NM genotoxicity testing, most often recognized for the comet assay but presumably important for any assay for genotoxicity testing of NMs, is accounting for potential photoactivation (Landsiedel, Ma-Hock, Van Ravenzwaay, et al., 2010 and references therein). The team of Schins (Gerloff et al., 2009) observed that TiO_2 induced DNA damage under normal laboratory light but not in the dark, as visualized in the comet assay. This was confirmed by Di Bucchianico et al. (2017) using anatase TiO_2, while Fenoglio et al. (2009) showed that rutile TiO_2 exhibited similar reactivity in the dark and under illumination. Specifically, TiO_2 can be activated with visible light close to UV (i.e., ≤413 nm), leading to the photocatalytic generation of ROS, which, in turn, can cause DNA damage (Dhandapani et al., 2012).

2.10 | Uptake of NMs into cells and into the nucleus

The transport of NMs into cells and into the nucleus is a complex process depending on many factors, one of the most important factors being the size of the NM (Landsiedel et al., 2012). The frequently used uptake routes into non-phagocytic mammalian cells, clathrin- or caveolin-dependent endocytosis, are limited to materials <80 nm (Drescher et al., 2011), excluding most aggregates. Extremely small NMs can passively permeate membranes (Schulz et al., 2012). Passive diffusion through nuclear pores for contact of the NM with nuclear DNA is restricted to molecules ≤8–10 nm in diameter (Magdolenova et al., 2014), excluding most NMs. However, larger NPs up to 39 nm can be transported into the nucleus through the nuclear pore complex via a signal-dependent mechanism (Panté & Kann, 2002). The latter mechanism is therefore expected to also allow reasonably small NM aggregates to find their way into the nucleus. Moreover, the nuclear membrane breaks down during mitosis, allowing NMs access to DNA depending on cell proliferation and on whether the exposure time is actually long enough to allow mitosis to occur (Azqueta & Dusinska, 2015; Karlsson, 2010).

In conventional cell cultures, the rate of uptake into cells is increased by the higher sedimentation rate of heavier NPs, leading to faster availability to the cells on the bottom of the culture dishes (the sedimentation rate of an NP is proportional to the square of its diameter; hence, a 10-fold difference in the diameter of an NP is expected to lead to a 100-fold difference in the sedimentation rate; DeLoid et al., 2017). Systems were therefore developed to eliminate or reduce this influence, such as inverted cultures, fluid flow or microfluidic systems (for details see Joris et al., 2013).

The uptake of the test article into the tester cells in vitro and into the tissue in vivo should be tested, especially if the assay has yielded negative results. This is most frequently done using TEM. It must be kept in mind that this method

can create a "drag-and-drop" phenomenon due to membrane destruction followed by sectioning into ultrathin slices. Raman imaging is an alternative that does not require sectioning (Ahlinder et al., 2013). Uptake can also be measured by SEM, confocal and fluorescence microscopy, Raman spectroscopy, side scatter (SSC) intensity analysis by flow cytometry, or reflection-based imaging. Recently, the use of laser ablation ICPMS (LA-ICPMS) has been reported for quantification of the association of nanomaterials with individual cells (OECD Draft Guidance Note, 2021).

The degree of uptake of the tested NM into the cells may also depend on the coating of the NM. Ivask et al. (2015) observed a higher increase in MNs with branched polyetherimide-coated Ag NPs than with citrate-coated Ag NPs. They suggested that the underlying reason was a stronger binding of the former to the tester cells leading to a larger uptake.

The uptake of the tested NM into the tester cells, according to the analysis by Elespuru et al. (2018) of in vitro comet assays on metal oxides and carbon nanotubes published from 2012 to 2014, was measured only in 9 out of 24 assays and, in publications on MN tests from 1997 to 2014, in only 34 out of 79 cases.

2.11 | Metabolism

Since NMs are usually not modified by xenobiotica-metabolizing enzymes, there is no stringent need to add S9 to in vitro tests on NMs unless the NM is composed of or likely to leach organic material that may be subject to mammalian xenobiotic metabolism. NMs composed (partially) of organic material may fit into xenobiotica-metabolizing enzyme binding pockets and undergo surface modification.

2.12 | Time frame

Quite generally, the time frame of exposure to the tested NM should be long enough to guarantee the possibility of direct contact with the nuclear DNA during mitosis when the nuclear membrane has vanished. Longer exposures at the same dose × time product led to much weaker or no MN induction (tested for five NMs: TiO_2, ZnO, Ag, $BaSO_4$, and CeO_2) compared with a higher dose × shorter time exposure (Llewellyn et al., 2021). This may be due to repair processes that operate over time, to threshold concentrations for secondary genotoxicity, to the formation of larger aggregates at higher concentrations, and/or to additional or unknown effects. The lower dose × longer time more realistically mirrors most human exposure scenarios.

2.13 | Grouping and read-across

Testing NMs for genotoxicity may not be the only or sometimes even the best and most efficient way to generate sufficient hazard information. Grouping is the general approach for assessing more than one chemical at the same time based on their similarity (OECD, 2014). Read-across predicts the toxicological endpoint of one chemical (target chemical) on the same endpoint for another chemical (source chemical), which is considered to be similar based on a scientific justification (OECD, 2014). Grouping and read-across have been adopted by REACh (European Union, 2006). A common hazard and risk assessment for sets of similar nanoforms of the same substance is already foreseen within REACh (European Commission, 2018; Janer, Ag-Seleci, et al., 2021; Janer, Landsiedel, & Wohlleben, 2021). The grouping of NMs of different compositions for the purpose of read across is generally foreseen in REACh; concepts for such grouping of NMs have been elaborated and should be used for assessing NM genotoxicity (Arts et al., 2014; Arts, Hadi, et al., 2015; Arts, Irfan, et al., 2015.

3 | COMET ASSAY

The comet assay, also called the single-cell gel electrophoresis assay, is the most frequently used genotoxicity test for NMs (as analyzed by Azqueta & Dusinska, 2015). It basically consists of separating intact from fragmented DNA by agarose gel electrophoresis. Undamaged supercoiled DNA remains at the origin, while broken or relaxed DNA migrates toward the anode, such that a tail is formed that resembles a comet. Visualization is usually performed using fluorescent dyes, although nonfluorescent staining with silver nitrate was reported to increase sensitivity and reproducibility

(Vandghanooni & Eskandani, 2011). The tail length and intensity are indicators of the level of DNA damage caused by the insult. The relative tail intensity (relative to tail plus head) yields a linear correlation with DNA breaks (Collins, 2004).

The comet assay has several advantages, such as high sensitivity (Kawaguchi et al., 2010; Vandghanooni & Eskandani, 2011), a low requirement for the number of cells, the possibility of being carried out in several cell types, and speed and ease of performance (Cardoso et al., 2021). It is therefore very frequently used for investigating potential DNA lesions produced by NM (Tables 1 and 2 shown for Ag NM). Nevertheless, the comet assay also has several shortcomings, such as rather low specificity and limited relevance to mutagenicity, since the DNA damage seen in the comet assay may be repaired before it is fixed as a mutation.

The comparison of positive versus negative NM genotoxicity tests in our 2009 review (Landsiedel et al., 2009) showed that (among the then relatively low number of NM genotoxicity tests) the comet assay was positive in 14 out of 19 studies (74% positive), whereas for the MN test, 12 out of 14 studies were positive (86%). The number of tests has since increased dramatically. A more recent statistic by Charles et al. (2018) on genotoxicity tests performed on the presumably most frequently tested NM, TiO_2, showed that in vitro Comet assays were positive in 63% of all *cases*, while MN tests were positive in 35% of cases (and CA tests in 20%, but only 5 CA tests were considered). An analysis by Wani and Shadab (2020) of genotoxicity studies on TiO_2 NPs from 2015 to the end of 2019 determined that among 19 studies, 11 showed positive in vivo comet tests (58%), 6 negative (32%) and 1 questionable (5). The evaluation of in vitro methods for human hazard assessment applied in the OECD Testing Program for the Safety of Manufactured Nanomaterials (OECD, 2018) concluded that "with the Comet assay, the responses were largely negative or equivocal for most of the investigated NM." The analysis of the in vivo genotoxicity of Ag NMs by Rodriguez-Garraus et al. (2020) showed that the comet assay gave fewer positive results than the MN and CA assays. Thus, experiences with the use of the comet assay for NMs vary from mostly positive to mostly negative.

The comet assay can be performed under several conditions (for a detailed description of the procedures, see Azqueta et al., 2013). If the electrophoresis step is performed at neutral pH, DNA double-strand breaks lead to fragments that move toward the anode, while under highly alkaline conditions (pH > 13) (by now the standard condition), the DNA double strand separates, and single-strand breaks also lead to free fragments that move ahead (more recent research has shown that this textbook association of the two kinds of breaks with the two pH conditions does not hold in a sharp way, but is actually overlapping, i.e., the association is preferential rather than absolute; Cordelli et al., 2021). The standard alkaline version (pH > 13) also detects alkali-labile sites (thus, double-strand breaks, single-strand breaks, and alkali-labile sites are all detected).

A further version, the comet assay in the presence of endonucleases (bifunctional DNA glycosylases/apurinic/apyrimidinic [AP] lyases), allows the detection of oxidative DNA base lesions. These enzymes cleave the oxidatively damaged DNA bases from the DNA core, leading to an apurinic or apyrimidinic site where their lyase (endonuclease) activities cleave the DNA. The resulting DNA break products are then measured by the comet assay.

Formamidopyrimidine glycosylase (Fpg) selectively recognizes oxidized purines (8-oxoGua [8-oxo-7,8-dihydroguanine], FaPyGua [2,6-diamino-4-hydroxy-5-formamidopyrimidine], FaPyAde [4,6-diamino-5-formamidopyrimidine] and other ring-opened purines), while EndoIII recognizes oxidized pyrimidines (thymine glycol and uracil glycol) (Collins et al., 1996). The premutagenic lesion 8-oxoGua is probably the most important lesion recognized by the most frequently used glycosylase in this modified comet assay, Fpg. In addition, there are further, less frequently used glycosylases. Alk A (3-methyladenine DNA glycosylase II) selectively recognizes DNA alkylation damage. T4 endonuclease V selectively recognizes DNA damage induced by UV (Liao et al., 2009). 8-Oxoguanine DNA glycosylase (HOGG1) has been found to recognize oxidative DNA lesions even more specifically than FPG and EndoIII (Smith et al., 2006).

The detection of oxidative DNA lesions by this modification of the comet assay does not yield essential information in addition to the information obtained by the standard alkaline comet assay, but the additional information is highly useful, especially when both methods, the standard alkaline comet assay and the comet assay, are used in the presence of one of the abovementioned endonucleases, which allows us to determine the relative contribution of oxidative DNA damage. This presents an approach to discriminate primary from secondary genotoxicity, although a sharp distinction is not straightforward in practice.

Kain et al. (2012) showed that Ag nanoparticles (NP) interact with the SH group at the active site of Fpg and thereby reduce the enzyme activity, but Magdolenova et al. (2014) reported that under the actual conditions of the assay, this does not significantly disturb the comet assay in the presence of Fpg. This conclusion was confirmed by the analysis of data on the genotoxicity of Ag NM by Rodriguez-Garraus et al. (2020).

TABLE 1 In vitro comet assay on sliver nanoparticles

AgNPs (nm)	Coating	Cells	Exposure (h)	Dose µg/ml*	Std	Fpg	Endo-III	OGG-1	References
5	bPEI + PVP	HABEC	48	1–20	+				Lebedová et al. (2018)
		HepG2	24	0.1–16	+	+	−		Ávalos et al. (2015)
		HL-60			+	+	+		
		NHDF			+	+	+		
		HPF			+	+	+		
5–15	PVA	Blood cells	4	1–50 (µM*)	+				Martinez Paino and Zucolotto (2015)
		HepG2			+				
5–20	AOT	HepG2	24	1–10	+				Brkic Ahmed et al. (2017)
	CTAB				+				
	PVP				+				
	BSA				+				
	PLL				+				
10	Nc	CACO2	24	1–50	−	+			Vila et al. (2018)
		CHO-K1	24	0.025–2.5	+				Souza et al. (2016)
		CHO-XRS5			+				
		JURKAT E6-1	24	2.5–20	+				Butler et al. (2015)
		TPH-1		1–25	+				
	PVP	BEAS-2B	4	10	−				Gliga et al. (2014)
			24		+				
	Citrate		4		−				
			24		+				
10–30		MCF-7	24	5–150	+				Farah et al. (2016)
13–60	Citrate	PK15	24	1–75	+				Milić et al. (2015)
			48		+				
15	Nc	MCF-10A	24	5.9–23.5	+	−			Roszak et al. (2017)
		MCF-7		4.1–16.3	+	−			
		MDA-MB-231		1.2–4.9	−	−			
		TK6	3	0.14–0.42	−	+			El Yamani et al. (2017)
				1.4–4.2	−	+			
				14	+	+			
			24	0.14–1.4	−	+			
				4.2	+	+			
				14	x	x			
		A549	3	0.01–1 µg/cm^2	−	−			
				3–10 µg/cm^2	+	+			
			24	0.01 µg/cm^2	−	−			
				0.03–3 µg/cm^2	−	+			
				10	+	+			
20	Nc	JURKAT E6-1	24	5–25	+				Butler et al. (2015)
		TPH-1		5–40	+				
	Nc	HepG2		20–160	+				Wang et al. (2019)

(Continues)

TABLE 1 (Continued)

AgNPs (nm)	Coating	Cells	Exposure (h)	Dose µg/ml*	Std	Fpg	Endo-III	OGG-1	References
	PVP				+				
20–50	Nc	HepG2	24	12.5–200	+				Che et al. (2017)
		A549			+				
		HepG2	48	1.5–200	+				
		A549			+				
27	Nc	NIH3T3	6	30.1–90.1	+				Jiravova et al. (2016)
		SVK14		25.4–76.1	−				
30	Citrate	HaCat	24	10–40	+				Bastos et al. (2017)
			48		+				
35	PVP	HeLa	12	1.25–10	+				Juarez-Moreno et al. (2017)
			24		+				
		MDA-MB-231	12		+				
			24		+				
		MCF-7	12		+				
			24		+				
		HMEC	24	2.5–5	+				Castiglioni et al. (2014)
		ECFC			+				
		BMDC	12	0.03–1	+				Castro-Gamboa et al. (2019)
40	Nc	HepG2	24	0.1–6.7	+	+	+		Ávalos et al. (2015)
		HL-60			+	+	+		
		NHDF			+	+	+		
		HPF			+	−	−		
	Citrate	BEAS-2B	4	10	−				Gliga et al. (2014)
			24		+				
45	Nc	MCF-10A	24	5.9–23.5	1	1			Roszak et al. (2017)
		MCF-7			−	−			
		MDA-MB-231		2–8.1	−	−			
		HBEC	48	1–20	+				Lebedová et al. (2018)
60	Nc	HEK293T	24	10–40	+				Jiang et al. (2013)
60–105	Nc	A549	24	25	−				Hatipoglu et al. (2015)
75	Citrate	BEAS-2B	4	10	−				Gliga et al. (2014)
			24		+				
100	Nc	CHO-K1	24	0.025–2.5	+				Souza et al. (2016)
		CHO-XRS5			+				
	PVP	GMO7492	24	0.01–10	+			+	Franchi et al. (2015)
	Nc	JURKAT E6-1	24	10–50	−				Butler et al. (2015)
		TPH-1			−				
50	Nc	JURKAT E6-1	24	10–50	−				Butler et al. (2015)
		TPH-1			−				
		BEAS-2B	4	10	−				Gliga et al. (2014)
			24		+				
56.4	Nc	A549	24	10–50	+				Hatipoglu et al. (2015)

TABLE 1 (Continued)

AgNPs (nm)	Coating	Cells	Exposure (h)	Dose µg/ml*	Std	Fpg	Endo-III	OGG-1	References
			48		+				
105	Nc	NIH3T3	6	1.6	+				Asare et al. (2016)
		SVK14		2.1	+				
		BJ		2.2	+				
131.5	Nc	NIH3T3	6	1.4	+				
		SVK14		2.2	+				
		BJ		2.3	+				
508,8	PEG	2D HepG2 spheroids	24	1 µg/cm^2	−	−			Elje et al. (2020)
				3–30 µg/cm^2	+	−			
		3D HepG2 spheroids		1–10 µg/cm^2	−	−			
				30 µg/cm^2	x	x			

Abbreviations: AgNPs, silver nanoparticles; AOT, sodium bis(2-ethylhexyl)-sulfosuccinate; bPEI, branched polyetherimide; BSA, bovine serum albumin; CTAB, cetyltrimethylammonium bromide; Endo-III, endonuclease III modified comet assay; Fpg, formamidopyrimidine DNA glycosylase modified comet assay; *µg, treatment expressed in quantity; *µM, treatment concentration micromolar; Nc, no coating; OGG-1, oxyguanine glycosylase 1 modified comet assay; PEG, polyethylene glycol; PLL, poly-L-lysine; PVA, polyvinyl alcohol; PVP, polyvinylpyrrolidone; Std., standard (alkaline) comet assay; +, positive result; −, negative result; x, unclear.

Source: Adapted from Rodriguez-Garraus et al., 2020 and complemented with more recent data.

A variation of the comet assay, which scores a reduction in the migration of the DNA fragments, indicates DNA cross links (Pfuhler & Wolf, 1996). This is important for risk assessment since DNA cross links are direct primary DNA lesions, as opposed to the secondary lesions frequently produced by NM via inflammation-dependent reactive oxygen species (ROS) production, ultimately leading to oxidative DNA damage. These latter lesions clearly require a threshold dose sufficient to produce inflammation. In addition, the use of diagnostic enzymes such as proteinase K can discriminate DNA–protein crosslinks from DNA–DNA crosslinks. Klingelfus et al. (2019) successfully used this modification to show a substantial degree of DNA–protein crosslinks produced by nanosilver (20 nm, PVP-coated) and nanotitanium (anatase, nanopowder <25 nm) in RTG-2 cells.

The comet assay can be used in vitro and in vivo (the in vivo comet assay has an OECD Test Guideline: OECD TG 489). Compared with the second most frequently used genotoxicity test on NM, the micronucleus (MN) test, the comet assay has the advantage that it can be employed with every organ, including those that make first contact upon ingestion or inhalation, as well as those representing a specific interest for a specific problem. On the other hand, Charles et al. (2018) concluded, in a review by France for REACH on in vivo studies concerning one of the most frequently investigated NMs, TiO_2, that "most of the studies did not bring adequate evidence that the NPs actually reach the investigated organ and/or did not include positive control and/or was not performed according to internationally accepted protocols and/or via relevant route of exposure for humans. The inconsistent in vivo results do not allow France to conclude on the genotoxicity of TiO_2 NPs." Similarly, Elespuru et al. (2018) concluded that most of their analyzed positive in vivo comet assays refer to studies using overload concentrations of NMs that clearly led to inflammation. Relier et al. (2017) defined an overload dose of P25 TiO_2 at 200–300 cm^2 of lung deposition or 4.2 µl/kg for volume-based lung exposure dose for the rat lung (confirming earlier studies that found similar numbers for other chemicals [Tran et al., 2000, Pauluhn, 2014]). At higher doses, clearance is impaired, and inflammation is induced, leading to inflammation-dependent genotoxicity.

The NM concentrations tested in the in vitro comet assay should range from not cytotoxic to approximately 80% viability since higher toxicities may lead to DNA strand breaks and therefore to false positives (Huk et al., 2015). The selection of the cytotoxicity assay used is essential. The Trypan blue dye exclusion method, for example, is not suitable for cytotoxicity determination in the in vitro comet assay (Kimura et al., 2013; Shi et al., 2010). The detectability of genotoxic damage in the comet assay is restricted to a relatively narrow range of approximately 100 to several thousand breaks per cell. Higher levels of breaks saturate the assay and may in extreme cases lead to disappearance of the comet images, although such NM concentrations are too cytotoxic and therefore are not relevant for the comet test evaluation

TABLE 2 In vivo comet assay on silver nanoparticles

AgNPs (nm)	Coating	Animal model	Tissue	AR	Exposure	Dose mg/kg bw	ST	Std	Fpg	Endo-III	OGG-1	References
5	Nc	Swiss albino mouse	Liver	p.o.	Single dose	5–100	3 h	+				Kant Avasthi et al. (2015)
							1 d	+				
				p.o.	35 day	10–20	3 h	+				
8	Citrate	New Zealand white rabbit	Liver	i.v.	Single dose	0.5–5	7 d	+				Kim et al. (2019)
							28 d	+				
10	Nc	Sprague Dawley rat	Bone marrow	p.o.	5 day	5–100	0*	+				Patlolla et al. (2015a)
10–80	Silicone	B6C3F1 mouse	Liver	i.v.	3 day	25	3 h	−		+	+	Li et al. (2014)
15–100	PVP	B6C3F1 mouse	Liver	i.v.	3 day	25	3 h	−		+	+	Li et al. (2014)
20	Nc	Wistar rat	Bone marrow	i.v.	Single dose	5	1 d	−				Dobrzyńska et al. (2014)
							1 w	−				
							4 w	−				
						10	1 d	−				
							1 w	−				
							4 w	−				
20	Nc	C57BL/6 mouse	Lung	i.v.	Single dose	5	1 d	−	−			Asare et al. (2016)
							1 w	−	−			
			Liver				1 d	−	−			
							1 w	−	−			
			Testis				1 d	−	−			
							1 w	−	−			
		OGG1−/− C57BL/6 mouse	Lung				1 d	−	−			
							1 w	−	−			
			Liver				1 d	−	−			
							1 w	−	−			
			Testis				1 d	−	−			
							1 w	−	−			
90	Nc	Wistar rat	Blood	p.o.	45 day	0.5	0*	−				Martins Jr et al. (2017)
			Liver				0*	−				
200	Nc	Wistar rat	Bone marrow	i.v.	Single dose	5	1 d	−				Kim et al. (2019)
							1 w	−				
							4 w	−				
		C57BL/6 mouse	Lung	i.v.	Single dose	5	1 d	−	−			Asare et al. (2016)
							1 w	−	+			
			Liver				1 d	−	−			
							1 w	−	−			
			Testis				1 d	−	−			
							1 w	−	+			
			Lung				1 d	−	−			
							1 w	+	−			
		OGG1−/− C57BL/6 mouse	Liver				1 d	−	−			
							1 w	−	−			
			Testis				1 d	−	−			
							1 w	−	−			

Abbreviations: Ag NPs, silver nanoparticles, size given in nm; AR, administration route; bw, body weight; d, day posttreatment; Endo-III, endonuclease III modified comet assay; Fpg, formamidopyrimidine DNA glycosylase modified comet assay; i.v., intravenous; Nc, no coating; OGG1−/−, 8-oxyguanine glycosylase knockout mouse; p.o., oral once a day; PVP, polyvinylpyrrolidone; ST, sampling time; Std, standard (alkaline) comet assay; w, week post treatment; 0*, day of finishing treatment; +, positive result; −, negative result.
Source: Adapted from Rodriguez-Garraus et al., 2020 and complemented with more recent data.

(Huk et al., 2015). According to present experience in the in vitro Comet assay, two- to sevenfold increases in % comet tail DNA may be expected upon treatment with Comet-active NM (Elespuru et al., 2018).

It must be kept in mind that the in vitro comet assay visualizes DNA damage, which later may be repaired (Karlsson, 2010). However, it may be reassuring that the reports on comet assays compared with MN assays on the frequently examined TiO_2 NM, if performed in parallel in the same study, overwhelmingly yielded the same results (Demir et al., 2015; Di Bucchianico et al., 2017; Proquin et al., 2017b; Roszak et al., 2013; Shukla et al., 2011, 2013; Stoccoro et al., 2016; Valdiglesias et al., 2013), with just two exceptions that gave a positive result for early DNA damage in the comet assay but no increase in MN (Armand et al., 2016; Guichard et al., 2012).

Several examples show a time-dependent drastic decrease in NM activity in the comet assay due to damage repair or possibly due to inflicted cells dying and being removed: El Yamani et al. (2017), Di Bucchianico et al. (2017), and Zijno et al. (2015) (such time-dependence is observed not only for NMs but also for other chemicals).

Several methods have been developed or successfully used for determining Comet measurement in a high- or semi-high-throughput fashion: Azqueta et al. (2013); Di Bucchianico et al. (2017); Dusinska & Tran (2015); El Yamani et al. (2017); Garcia-Rodriguez et al. (2019); Gutzkow et al. (2013); Harris et al. (2015); Huk et al. (2015); Nelson et al. (2017); Rubio et al. (2016); Stang & Witte (2009); and Watson et al. (2014). Software for image analysis, such as Metafer or MetaCyte CometScan, reduces the analysis time compared with manual fluorescence analysis from hours to minutes.

Several possibilities for the interaction of the tested NM with the comet assay should be considered. If the NM is present in the comet head, this may interfere with the scoring (Di Bucchianico et al., 2017). The presence of NMs during the electrophoresis step may lead to additional DNA damage and may influence migration (Azqueta & Dusinska, 2015; Charles et al., 2018). In the modified Comet versions, which include endonucleases, NM may influence the activity of these enzymes by steric hindrance, induced conformation changes, electrostatic interaction or hindrance of enzymes from reaching the damaged DNA (Kain et al., 2012). However, for balance, it must be added that several authors maintain that under standard conditions it is unlikely that any of the interferences mentioned in this paragraph will fundamentally falsify results obtained using the comet assay for investigating the potential genotoxicity of NMs (Azqueta & Dusinska, 2015; Di Bucchianico et al., 2017; Karlsson, 2010).

The comet assay yields very frequently positive results on NMs. Several conceivable reasons for artifactually positive results in the comet assay on NM have been postulated (Karlsson et al., 2015) or shown (Ferraro et al., 2016). Not very long ago, Elespuru et al. (2018) went to the point of stating regarding the in vitro comet assay: "Because of a lack of standard methods and uncertainty over the meaning of results, the Comet assay is not recommended as a screening assay for NM genotoxicity assessment." Nevertheless, the in vitro comet assay is still the most frequently used genotoxicity test on NM for sound reasons discussed at the beginning of this chapter. With respect to the in vivo version of the comet assay, Elespuru et al. (2018) stated that, although not developed for use with NM, the OECD TG 489 "In Vivo Mammalian Alkaline Comet Assay" can also be used for NM without a need for modifications, that is, basic performance conditions, such as lysis buffer, unwinding, electrophoresis parameters, and so on, may be used in the same way as for testing larger bulk materials.

For the in vitro comet assay, there are no validated OECD guidelines, and the approaches taken thus far are very diverse.

4 | MICRONUCLEUS TEST

Micronuclei (MN) are formed during the anaphase of the cell cycle from lagging chromosomes or chromosome fragments occurring after chromosome lesions (structural change, clastogenicity) or after chromosome malsegregation (numerical change, aneugenicity).

Briefly, the in vitro form of the test is performed by exposing cell cultures to the test material with and without an exogenous source of xenobiotic metabolism. Exposure is maintained for a period of time, allowing chromosome damage to occur, leading to the formation of micronuclei in interphase cells, which are visualized microscopically after staining. Micronuclei should only be scored in cells that complete nuclear division following exposure. Cell proliferation must be demonstrated, and cytotoxicity must be assessed.

The in vivo form of the test is performed by exposing animals to the test material. After sufficient time for the detection of treatment-related induction of micronucleated immature erythrocytes, the bone marrow is extracted or blood is collected, followed by staining and analysis for the presence of micronuclei by microscopy, image analysis, flow cytometry, or laser scanning cytometry.

TABLE 3 In vitro micronucleus tests on sliver nanoparticles

AgNPs (nm)	Coating	Cells	Exposure (h)	Dose µg/ml*	Results	References
5	Nc	TK6	28	1–1.5	+	Li, Qin, et al. (2017)
		HBEC	48	1–20	–	Lebedová et al. (2018)
10	Nc	CHO-K1	24	0.025–2.5	+	Souza et al. (2016)
		CHO-XRS5			–	
		JURKAT E61	24	1–25	+	Butler et al. (2015)
		TPH-1			+	
15	Nc	MCF-10A	24	5.9–23.5	–	Roszak et al. (2017)
		MCF-7	48	4.1–16.3	–	
		MDA-MB-231		1.2–4.9	–	
		CHO-K1	24	1–5–10	+	Jiang et al. (2013)
20	Nc	JURKAT	24	1–25	+	Butler et al. (2015)
		TPH-1			+	
		HepG2	48	1–10	+	Sahu et al. (2014)
		CACO2			–	
		HepG2		5–15	–	
		CACO2			–	
20	Nc	HepG2	24	20–160	+	Wang et al. (2019)
	PVP				+	
	Citrate	JURKAT	24	0.1–25	+	Ivask et al. (2015)
	bPEI				+	
	Citrate	WIL-2-NS			+	
	bPEI				+	
	Citrate				+	
	bPEI	Lymphocytes	44	0.2–25	+	Guo et al. (2016)
	Citrate	L5178Y	4	1.25–4	+	
		TK6		2.5–15	+	
	PVP	L5178Y		1.25–8	+	
		TK6		2.5–30	+	
20–50	Nc	HepG2	24	12.5–200	+	Wang et al. (2017)
		A549			+	
		HepG2	48	12.5–200	+	Che et al. (2017)
		A549			+	
30	Citrate	HaCat	24	10–40	+	Bastos et al. (2017)
			48		+	
30	PVP	Humane 3D HepG2 spheroids	24	0.2	–	Llewellyn et al. (2021)
				2	–	
				10	–	
				0.5	+	
				1	+	
				5	+	
			120	0.2–10	–	
45	Nc	MCF-10A	24	5.9–23.5	–	Roszak et al. (2017)
		MCF-7	48	4.1–16.3	–	

TABLE 3 (Continued)

AgNPs (nm)	Coating	Cells	Exposure (h)	Dose µg/ml*	Results	References
		MDA-MB-231		1.2–4.9	−	
50	Nc	HABEC	48	1–20	−	Lebedová et al. (2018)
		HepG2	4	10–100	−	Sahu, Roy, et al. (2016)
			24	2.5–25	−	
		CACO2	4	10–100	−	
			24	2.5–25	−	
		HepG2	40–44	1–20	+	Sahu, Njoroge, et al. (2016)
		CACO2	24	1–50	−	Butler et al. (2015)
		JURKAT			+	
		TPH-1			+	
50	Citrate	L5178Y	4	1.25–20	+	Guo et al. (2016)
		TK6		2.5–120	−	
		L5178Y		1.25–30	+	
	PVP	TK6		2.5–120	−	
90	Nc	Balb3T3	24	0.17–10.60	+	Choo et al. (2017)
		A31-1-1			+	
100	Nc	CHO-K1	24	0.025–2.5	−	Souza et al. (2016)
		CHO-XRS5			+	
		JURKAT		1–50	+	Butler et al. (2015)
		TPH-1			+	
	Citrate	L5178Y	4	1.25–35	+	Guo et al. (2016)
		TK6		2.5–400	+	
	PVP	L5178Y		1.25–50	+	
		TK6		2.5–400	+	
100–200	Nc	BEAS-2B	48	1	+	Cervena et al. (2021)
				10	+	

Abbreviations: AgNPs, silver nanoparticles; size given in nm; bPEI, branched polyetherimide; nc, no coating; PVP, polyvinylpyrrolidone; +, positive result; −, negative result.
Source: Adapted from Rodriguez-Garraus et al., 2020 and complemented with more recent data.

The use of centromere fluorescence in situ hybridization or kinetochore staining allows discrimination between clastogenic and aneugenic modes of NM action (Fenech, 2006; Pfuhler et al., 2013). This enables the demonstration of direct contact between the investigated NM and chromatin.

Clastogenicity can be seen in the MN and in the chromosomal aberration (CA) assay (see next chapter). The MN assay has several advantages. In the MN assay, aneugenicity can readily be seen, while in the CA test, polyploidy can be seen, but not necessarily aneugenicity. Moreover, the MN test is faster, easier, and less subjective than the CA test. Because of these positive qualities and on the grounds of its sensitivity, the MN test is very frequently used for detecting potential chromosome mutations formed by NMs (Tables 3 and 4 for Ag NMs).

The in vitro MN assay has been shown to be remarkably well suited to discriminate between linear and nonlinear dose–response relationships (Doak et al., 2009; Elhajouji et al., 1998), which is useful for evaluating the biological relevance of the results.

A frequently used variation of the MN test is the cytokinesis-block MN test (CBMN). In this variation, the actin assembly inhibitor cytochalasin B is used to inhibit cell division after mitosis. This leads to the formation of binucleated cells, which allows restricting the scoring for MN to cells that have undergone mitosis during or after treatment with the test article.

TABLE 4 In vivo micronucleus tests on sliver nanoparticles

AgNPs (nm)	Coating	Animal model	Tissue	AR	Exposure	Dose mg/kg bw	ST	Result	References
5	PVP	B6C3F1 mouse	Blood	i.v.	Single dose	0.5–20	2 d	–	Li et al. (2014)
6.3–629	Nc.	Sprague Dawley rat	Bone marrow	i.v.	Single dose	5	1 d	–	Wen et al. (2017)
10	Nc	Sprague Dawley rat	Bone marrow	p.o.	5 days	5–100	1 d	+	Patlolla et al. (2015a, 2015b)
10–80	Silicone	B6C3F1 mouse	Liver	i.v.	Single dose	25	2 d	–	Li et al. (2014)
					3 days			–	
15–100	Nc	B6C3F1 mouse	Liver	i.v.	Single dose	25	2 d	–	Li et al. (2014)
	PVP							–	
20	Citrate	C57BL/6 mouse	Blood	p.o.	3 days	4	1 d	+	Nallanthighal, Chan, Bharali, et al. (2017)
					7 days			+	
		OGG1−/− C57BL/6 mouse			3 days			+	
					7 days			+	
20	Citrate	C57BL/6 mouse	Blood	p.o.	7 days	4	1 d	+	Nallanthighal, Chan, Murray, et al. (2017)
							1 w	+	
							2 w	+	
	PVP						1 d	–	
							1 w	–	
							2 w	–	
	Nc	Wistar rat	Bone marrow	i.v.	Single dose	5	1 d	+	Dobrzyńska et al. (2014)
							1 w	+	
							4 w	–	
						10	1 d	+	
							1 w	+	
							4 w	+	
	Nc	ICR mouse	Bone marrow	p.o.	28 days	10–250	1 d	+	Wang et al. (2019)
	PVP							+	
200	Nc	Wistar rat	Bone marrow	i.v.	Single dose	5	1 d	+	Dobrzyńska et al. (2014)
							1 w	+	
							4 w	–	

Abbreviation: AgNPs, silver nanoparticles size given in nm; AR, administration route; bw., body weight; d, day post treatment; i.v., intravenous; Nc, not coating; OGG1−/−, 8-oxoguanine glycosylase knockout mouse; p.o., oral once a day; PVP, polyvinylpyrrolidone; ST, sampling time; w, week post treatment; +, positive result; −, negative result.

Source: Adapted from Rodriguez-Garraus et al., 2020 and complemented with more recent data.

It is probably appreciated by most investigators that in the CBMN assay, the use of cytochalasin B simultaneously with exposure to the test article can inhibit the uptake of the latter by endocytosis into the tester cells, potentially leading to undetectable levels of genotoxicity of the tested NM (Karlsson, 2010). As an example, Doak et al. (2009) showed that only posttreatment with cytochalasin B allowed an increase in MN upon treatment of MCL-5 cells with dextran-UPSION, but neither cotreatment nor treatment with cytochalasin B ahead of treatment with NM. The test article should therefore be added some hours (ideally 24 h) prior to the addition of cytochalasin B. This allows the cells to go through a complete cell cycle in presence of the tested NM, including the phase during mitosis that gives the NM free

access to the nuclear DNA while the nuclear membrane is disintegrated. In addition, this time period also allows sufficient internalization of NMs with slow uptake.

A high content of NM aggregates/agglomerates in or adsorbed to the tester cells can interfere with the detection of MN, as shown, for example, for TiO_2 by Shukla et al. (2013). This problem can be circumvented by limiting the NM concentration to 100 μg/ml. At this level, the nanoparticles do not interfere with the scoring. Alternatively, flow cytometry, where cells are lysed and MNs and nuclei are individually analyzed instead of optical scoring, can be helpful (Gonzalez et al., 2014). Li, Qin, et al. (2017) observed a failure to detect MN induction by TiO_2 using fluorescence detection in the flow assay. They suggested that this may have been due to interference of the TiO_2 agglomerates with the fluorescence detection.

OECD specifically recommends some cell lines for use in the MN test (OECD 487, 2020) (in contrast to these recommendations, our experience shows that V79 cells [despite their good uptake potential] are not appropriate for NM testing [OECD Draft Guidance Note, 2021]). Standard cell lines possessing suitably low spontaneous MN formation are recommended (Lorge et al., 2016). If the MN test is performed using cells in culture, it is critical to take into account their genomic (karyotype) stability, DNA repair capacity and p53 status (for details see Dusińska et al., 2019).

The presence/concentration of serum in the medium used for the in vitro MN test appears to be critical for the outcome but not in a uniformly simple way. Lindberg et al. (2009) observed no significant induction of MN upon treatment of BEAS-2B cells with single-walled carbon nanotubes in the absence of serum, while Doak et al. (2009) found an increase in MN upon treatment of BEAS-2B cells with single-walled carbon nanotubes only in the presence of 2% but not 10% serum. However, in the study by Prasad et al. (2013), the outcome was positive only at 10% FBS, the highest of three concentrations tested. The authors suggested that the high serum concentrations led to smaller aggregates of NM and TiO_2, facilitating their uptake into the tester cells.

Vecchio et al. (2014) developed a lab-on-a-chip system including automated image analysis for a high-throughput CBMN assay that successfully showed high genotoxicity of silver NPs in primary human lymphocytes.

High-throughput flow cytometry versions of the MN test have been developed and/or successfully used, allowing the assessment of a large number of cells (Avlasevich et al., 2011; Bryce et al., 2013; Decordier et al., 2009; Di Bucchianico et al., 2017; Fenech et al., 2013; Gea et al., 2019; Laban et al., 2020; Niu et al., 2016; Platel et al., 2016; Roemer et al., 2015). A detailed description of a flow cytometry/MN assay, including delineation of the confounding factors, restrictions and limitations, can be found in Nelson et al. (2017).

For the in vivo MN test, the route of application appears to play an important role. In vivo investigations of the potential genotoxicity of Ag NM in the MN assay were positive after intravenous or intraperitoneal administration (Landsiedel, Ma-Hock, Kroll, et al., 2010, Ghosh et al., 2012, Dobrzyńska et al., 2014 (as a limitation of the latter study: rat MN testing without separating the nucleated cells could be misleading, especially if the NM can cause mast cell degranulation). However, peroral treatment (dosed up to 1000 mg/kg for 28 days with Ag of a similar size: 52–71 nm) led to negative results (Kim et al., 2008). This is consistent with the predominant experience that peroral exposure to NM does not represent a major route of genotoxic threat by NM.

The OECD TG 487 "In Vitro Micronucleus Assay", albeit not developed for use with NM, may also be used for testing NMs, taking into account the modifications and precautions discussed above. An OECD draft GD for its adaptation to testing NMs is available (OECD DRAFT Guidance Notes on the Adaptation of the In Vitro Micronucleus Assay [OECD TG 487] for Testing of Manufactured Nanomaterials; OECD, 2021). The OECD TG 474 on the in vivo MN test ("Mammalian Erythrocyte Micronucleus Test"), albeit also not developed for use with NM, can be used for this purpose (Elespuru et al., 2018), but, of course, the systemic availability of the tested NM has to be demonstrated.

5 | CHROMOSOMAL ABERRATION TEST

The CA test detects chromosome and chromatid breaks and other chromosome damage such as translocations (clastogenicity) as well as alterations in the number of chromosomes (polyploidy) (polyploidy alone does not prove aneugenicity but could also stem from cell cycle perturbation or cytotoxicity; hence, aneugenicity is better proven by MN assay). These defects are microscopically scored in the metaphase of the cell cycle after a metaphase-arresting substance such as colcemide or colchicine has led to accumulation of cells in the metaphase (Aoshima et al., 2010). Table 5 shows CA tests on Ag NM as an example.

TABLE 5 In vivo chromosome aberration test on silver nanoparticles

AgNPs (nm)	Coating	Animal model	Tissue	AR	Exposure	Dose mg/kg bw	ST	Result	References
10	Nc.	Sprague Dawley rat	Bone marrow	p.o.	5 day	5–100	1 d	+	Patlolla et al. (2015a)
		Albino rat	Bone marrow	i.p.	28 day	1–4	0*	+	EI Mahdy et al. (2015)
6–629	Nc.	Sprague Dawley rat	Bone marrow	i.v.	Single dose	5	1 d	+	Wen et al. (2017)

Abbreviation: AgNPs, silver nanoparticles, size given in nm; AR, administration route; bw, body weight; d, day post treatment; i.v., intravenous; Nc, no coating; p.o., oral once a day; ST, sampling time; 0*, day of termination treatment; +, positive result; −, negative result.
Source: Adapted from Rodriguez-Garraus et al., 2020.

Catalan et al. (2011) reported that CA was maximal after 48 h of treatment of human lymphocytes with NMs (multiwalled carbon nanotubes) and concluded that treatment with NMs for approximately two cell cycles is ideal.

It appears that there is a general consensus that doses of NM above 500 μl/ml in the CA test are excessive.

None of the five studies that included S9 in the analysis by Elespuru et al. (2018) indicated that S9 led to a different result. Only two out of the 11 analyzed CA tests included a measurement of the uptake of the investigated NM into the tester cells, which represents an important issue when negative results are obtained. These two studies showed that the NM was internalized (Di Giorgio et al., 2011; Hackenberg et al., 2011).

Although not developed for NM investigation, the OECD TG 475 "Mammalian Bone Marrow Chromosomal Aberration Test" can be used for NMs (Elespuru et al., 2018); however, appropriate adaptations of the protocol might be needed. The authors stipulate as necessary adaptations for its use with NMs that "attention should be given to mechanistic aspects such as relevant tissue exposure and potential particle overload effects."

6 | AMES TEST

The Ames test is a fast method for scoring gene mutations. It is based on the appearance of colonies formed from amino acid-requiring mutants of *Salmonella typhimurium* or *Escherichia coli* in agar deficient in the amino acid required by the mutant tester strain used. These colonies arise from back mutations of the tester strains, which initially carry mutations in genes required for the synthesis of the respective amino acids.

Briefly, suspensions of bacterial cells are exposed to the test material in the presence and absence of an exogenous xenobiotica-metabolizing system (usually Aroclor 1254-induced rat liver S9). After incubation for 2 or 3 days, revertant colonies are counted. While most tests scoring for NM-induced genotoxicity lead to considerably great numbers of apparently genotoxic NMs, the Ames test yields mostly negative results, as already shown in our early review (Landsiedel et al., 2009) and in numerous overviews since then (e.g., Doak et al., 2012; Magdolenova et al., 2014). Attempts to explain this negative behavior include possible penetration problems through the bacterial cell wall/cell membrane of Ames bacteria for many NMs, as already indicated in our early review (Landsiedel et al., 2009). Bacteria do not possess mammalian mechanisms for uptake into cells (endocytosis, pinocytosis, phagocytosis). The bacteria used in the Ames test were Gram-negative bacteria. Gram-negative bacteria possess porins in their outer membrane, which generally only allow hydrophilic materials (Galdiero et al., 2012) of less than 600 Da (Novikova & Solovyeva, 2009) to diffuse through them, while the basic structure of this outer membrane is a bilayer of lipopolysaccharides and phospholipids more rigid than most bilayers, which considerably slows the diffusion of lipophilic materials (Zgurskaya et al., 2015). In addition, the inner membrane of Gram-negative bacteria represents the major permeability barrier (Benz, 1989), which is too complex for extensive discussion in the frame of this review. Overall, the uptake of most NMs into the bacteria used in the Ames test appears to be substantially hindered, which is consistent with the experience that the Ames test genotoxicity investigations on NMs were negative in almost all cases.

Kumar et al. (2011) reported the uptake of NMs (ZnO and TiO_2) into *Salmonella typhimurium*, which was increased by the presence of S9, explained by the authors by a possible formation of micelles or by formation of a protein coat. The uptake occurred at nonbactericidal concentrations and was shown by transmission electron microscopy (TEM) and flow cytometry. NPs of up to 70 nm were observed in the cells, while agglomerates adhered outside the membrane.

A few studies reported positive results in the Ames test, either very weak (~2-fold increases: Lopes et al., 2012, Gomaa et al., 2013, Sadiq et al., 2015) or obtained under unacceptable conditions (starvation: Jomini et al., 2012, treatment in 55°C agar: Liu et al., 2014, background bacterial colonies for *E. coli* WP2 >200: Pan et al., 2010). Kumar et al.

(2011) also reported borderline positive results (~2-fold increase), which were not confirmed by Butler et al. (2014). Clift et al. (2013) found a weakly positive result with diesel exhaust, which was likely due to the leaching of mutagenic polycyclic aromatic hydrocarbons.

Although not very likely to be the reason for the mostly negative Ames tests on NM, additional problems may arise from the small size of the bacteria, which are not much larger than some MN and/or from the possibility that NM, if they do enter the bacterium, may interfere with histidine synthesis, possibly leading to false-positive or false-negative results (Dusinska et al., 2015). The proteins in S9 could in principle lead to interference with the uptake of some NMs into Ames bacteria such that tests performed with S9 alone could yield false negative results. However, reported changes in the presence versus absence of S9 (Gomaa et al., 2013; Kumar et al., 2011; Liu et al., 2014; Lopes et al., 2012) do not support such an assumption, since they did not yield consistent results (even not within a lab). Finally, and trivially, NMs with bactericidal/bacteriostatic properties (such as Ag NPs) obviously cannot be tested with the bacterial Ames test. Overall, Elespuru et al. (2018) conclude in their analysis, which covered all Ames tests on NM until the end of 2014, that they found "a lack of convincing validity for most if not all of the reported positives." A very recent study reported a statistically significant and dose-dependent but very weak positive Ames test with one of the four tested nano-SiO_2 forms ("SB," 22 nm) in one of the *S. typhimurium* strains (TA100) without S9 (Chen et al., 2021). Although the result appears minimally convincing, a clearer result using one of the mammalian gene mutation assays would be desirable. In a further very recent study on cellulose nanofibrils, the Ames test remained negative but could obviously not be adequately performed because the NM precipitated at 12.5 μg per plate. Moreover, it was not tested whether the NM entered the bacteria (Fujita et al., 2021).

It appears advisable to normally refrain from the use of the Ames test for scoring the potential mutagenicity of NMs and to routinely prefer to use mammalian cell mutation assays instead, as discussed in the subsequent chapter.

7 | MAMMALIAN GENE MUTATION ASSAYS

The in vitro *mammalian gene mutation using the thymidine kinase* (*Tk*) *gene* (OECD TG 490) is based on mutation of the autosomal *Tk* (thymidine kinase) gene and detects both gene and chromosome mutations as well as epigenetic gene silencing of the functional *Tk* allele due to promoter hypermethylation (Cheng et al., 2013). The standard *Tk* assay is performed in mouse lymphoma L5178Y/$Tk^{+/-}$ cells (MLA Assay), which have a mutated p53 gene. Large and small colonies can be distinguished. The large colonies arise due to small genetic damage (base-pair substitutions, small deletions) and therefore grow relatively fast. They indicate gene mutations. The small colonies arise due to large deletions, chromosome rearrangements, and mitotic recombination as well as specifically due to large damage to chromosome 11b, which harbors the *Tk* gene. They indicate clastogenicity.

Briefly, mouse lymphoma L5178Y/$Tk^{+/-}$ cells in suspension are exposed to the test material in the presence and absence of an exogenous xenobiotica-metabolizing system and then subcultured to determine cytotoxicity and to allow phenotypic expression followed by mutant selection. Mutant frequency was determined by seeding cells in medium containing the selective agent to detect mutant colonies and in medium without the selective agent to determine viability. Mutant frequency was calculated using the number of mutant colonies corrected by the cloning efficiency at the selection time.

The MLA test can be used as a fluctuation assay with 96-well plates and trifluorothymidine as a selection marker. Table 6 shows the MLA on Ag NM as an example.

TABLE 6 In vitro mammalian mutation tests (MLA) on sliver nanoparticles

AgNPs (nm)	Coating	Cells	Exposure (hours)	Dose (μg/ml)	Results	Reference
20	Citrate	L5178Y	4	1–60	+	Guo et al. (2016)
	PVP				+	
50	Citrate				+	
	PVP				+	
100	Citrate				+	
	PVP				+	

Note: Results with $p < .05$ at any of the doses tested were considered as positive.

Abbreviations: AgNPs, silver nanoparticles; Dose, range of doses tested; MLA, mouse lymphoma assay; PVP, polyvinylpyrrolidone; +, positive result.

Source: Adapted from Rodriguez-Garraus et al. (2020).

There is a variation of the mouse lymphoma assay using p53-proficient human lymphoblastoid *TK6* cells.

The Hprt assay (OECD TG 476) is based on the mutation of the hypoxanthine guanine phosphoribosyltransferase gene, which is X-linked with only one active copy per cell, necessitating only one mutation for phenotypic expression. Similar to the MLA, it can detect gene mutations and deletions. However, the MLA detects deletions with higher efficiency (Johnson, 2012). The standard *Hprt* assay is performed in Chinese hamster V79 cells. Doak et al. (2012) warned that the use of cell lines with reduced DNA repair activity, such as WIL2-NS, could lead to false-positive results.

The principle of the test is similar to that described above for the mouse lymphoma assay.

The above indicated OECD TGs for these two tests are not written for NM testing but appear satisfactory for this use as well (Elespuru et al., 2018), of course with due attention as required for all genotoxicity tests on NM, such as characterization of the NM in the test milieu, securing uptake of the tested NM into the tester cells and appropriate cytotoxicity measurement (if the top concentration is based on cytotoxicity, the highest concentration used should aim to arrive at 10%–20% survival, while cytotoxicity leading to <10% survival is generally considered excessive). The HPRT assay according to OECD 476 describes only a 4 h treatment period. However, it is not clear whether this allows sufficient time for NM uptake.

Nonstandard mammalian cell gene mutation tests for NMs include *Hprt* assays in MCL-5 and WIL2-NS cells (Manshian et al., 2013), the *Aprt* (adenine phosphoribosyltransferase) assay in 3C4ES cells (Zhu et al., 2007), the transgenes (*lacZ* or *cII*) in FE1 cells (Jacobsen et al., 2008) and the loss of CD59 in AL cells (Wang et al., 2015).

The Pig-a gene mutation assay scores mutations in the X-linked phosphatidylinositol glycan anchor biosynthesis, class A gene (CD59 for the rat) (Krüger et al., 2015).

Briefly, for the in vivo version, animals are treated by a route allowing the exposure of the bone marrow. The frequency of CD59− (for rats) or CD24− (for mice) negative reticulocytes (RETs) and total red blood cells (RBCs) are determined. Since RET is a minor blood cell constituent, immunomagnetic separation is recommended prior to analysis (Dertinger et al., 2011). The standard procedure is an automatic flow cytometric analysis suitable for routine testing.

In principle, the Pig-a-gene mutation assay can also be performed in vitro (Bemis & Heflich, 2019). Using B-lymphoblastoid TK6 cells and CD55 and CD59 to differentiate between mutant and wild-type cells, a 4-h incubation with mutagens followed by a 10-day expression time and analysis by flow cytometry was found to be suitable (Olsen et al., 2017).

Cordelli et al. (2017) used the test successfully for chronic (6 h/day on 5 consecutive days/week for 2 years) in vivo (rat reticulocytes and erythrocytes upon whole-body exposure) investigation on a potential genotoxicity of several low doses (0.1, 0.3, 1, or 3 mg/m^3) of CeO_2 NM and a high dose (50 mg/m^3) of $BaSO_4$ NM. In agreement with other tests used in the same study (alkaline comet assay, MN test), no genotoxicity was observed, while the positive control (ethylnitrosourea) showed the expected genotoxicities.

Regarding in vivo gene mutation tests on NM, the analysis by Elespuru et al. (2018) showed that positive tests were generally considerably weaker (2- to 3-fold higher than the negative controls) than the same tests performed on chemicals of standard sizes, such as *N*-ethyl-*N*-nitrosourea or 7,12-dimethylbenz[a]anthracene, which are frequently used as standard controls. At least some of the mutation spectra observed in the gene mutation tests on NM indicated oxidative DNA damage (including G to T transversions), likely due to inflammation-dependent ROS generation.

The limited experience with the use of the Pig-a gene mutation assay for NM genotoxicity testing indicates that this test is suitable for investigating the potential genotoxicity of NM.

8 | GAMMA-H2AX FOCI

This test is based on the phosphorylation of the DNA-associated histone protein H2AX, a protein involved in the repair of DNA double-strand breaks. The phosphorylated form is called γ-H2AX. A focus of this modified histone protein forms close to a DNA double-strand break and can be detected by immunohistochemistry by using an antibody to this form that carries a fluorescent tag. The number of these foci is directly correlated with the number of DNA double-strand breaks (Kuo & Yang, 2008).

The advantages of this test include the simplicity of the procedure (Nelson et al., 2017) and high sensitivity (Kuo & Yang, 2008). The evaluation of in vitro methods for human hazard assessment applied in the OECD Testing Program for the Safety of Manufactured Nanomaterials (2018) concluded that the bright foci at the site of the DNA breaks make this method the most sensitive assay for detecting DNA damage. However, γ-H2AX levels vary substantially between cell lines and during the cell cycle, necessitating careful measurement of the background levels before the exposure of the cells to NMs. The time course of the appearance and disappearance of NM-induced γ-H2AX levels varied among individual NMs and cell lines. Time course studies are recommended (Nelson et al., 2017).

Double-strand breaks represent an especially severe form of genotoxicity, but thus far, relatively little information exists on the use of this test for scoring NM genotoxicity. It has, however, been used successfully for scoring DNA double-strand breaks produced by NM (e.g., Karlsson et al., 2014). A flow cytometric version of this test is also available (Lewis et al., 2010; Ismail et al., 2007). As generally observed when using flow cytometry with NM, there could be interferences in the analyses due to interference of NM agglomerates with the fluorescence detection.

The limited experience with the use of the gamma-H2AX foci assay for NM genotoxicity testing indicates that this test is suitable for investigating the potential genotoxicity of NMs.

9 | FLUORIMETRIC ANALYSIS OF DNA UNWINDING

Fluorimetric analysis of DNA unwinding (FADU) is based on progressive DNA unwinding (denaturation). At alkaline pH, this denaturation begins at DNA open sites including strand breaks but also chromosome ends or replication forks (with respect to DNA breaks measured by FADU representing background). The low fluorescence intensity of a dye bound to double-stranded DNA indicates a high degree of unwinding and hence (under equal background) a high degree of DNA strand breaks. The assay can be modified to detect DNA crosslinks or 8-oxo-2′-deoxyguanosine (8-oxo-dG) (Nelson et al., 2017). The assay can be performed in an automated 96-well plate high-throughput form and has been used successfully for demonstrating DNA strand breaks induced by NMs such as ZnO (Moreno-Villanueva et al., 2011). The fluorescence of NMs could interfere with the FADU test based on fluorometric analysis.

The limited experience with the use of the FADU assay for NM genotoxicity testing indicates that this test is suitable for investigating the potential genotoxicity of NM.

10 | TOXTRACKER ASSAY

The ToxTracker assay was initially designed for chemicals (Hendriks et al., 2012), adapted by Karlsson et al. (2014) and used by Åkerlund et al. (2018) for NM. It is a mechanism-based reporter assay using mouse embryonic stem cells and green fluorescent protein (GFP)-tagged markers. The cells are untransformed and proficient in all major DNA damage and cellular stress response pathways, and they have a high proliferation rate, making them sensitive to DNA damage. Two markers indicate oxidative stress (Srxnl and Blvrb reporters), two markers indicate DNA damage, one of them via the signaling pathway for replication stress (Bscl2 reporter), the other via NFKB signaling (Rtkn reporter), and two markers indicate general p-53-dependent cellular stress (Btg2 reporter) or protein unfolding (Ddit3 reporter). The cells were exposed to the NM for 24 h, followed by flow cytometry to detect fluorescence. The use of 96-well plates allows medium to high throughput (Brown et al., 2019; Karlsson et al., 2014). Interestingly, as reported by Nelson et al. (2017), none of the NMs investigated in a panel of metal oxides and silver NMs, diesel, carbon nanotubes, quartz and quantum dots clearly induced the Bscl2 reporter, indicating that in all likelihood, none of the investigated NMs could interact directly with DNA. The conventional assays (comet and γ-H2AX) obtained in this NM testing panel confirmed the results of the ToxTracker assay. McCarrick et al. (2020) also observed that most, albeit not all, of the NMs that they investigated (metals, metal oxides and quantum dots) did not activate reporters related to DNA damage but activated oxidative stress indicators. The following NMs clearly activated the Rtkn DNA damage reporter: CuO, Co, CoO, CdTe QDs, Mn, Mn_30_4, and V_20_5. Thus, it appears that this assay is useful to single out the relatively few NMs liable to lead to direct primary DNA damage. However, unresolved discrepancies between results in the ToxTracker and other assays to discover NM genotoxicity, such as the comet assay, have also been observed (most clearly in HepG2 cells) (Brown et al., 2019).

11 | DROSOPHILA WING SPOT TEST

An alternative in vivo model for NM genotoxicity testing is the use of a nonmammalian whole animal test in Drosophila. The relatively short reproductive cycles of Drosophila allow a fast test, which at the same time is open for high throughput (Pitchakarn et al., 2021). The Drosophila wing spot test (also called the somatic mutation and recombination test [SMART]) is based on the loss of heterozygosity in dividing wing cells during larval development and is easily recognized as mutant wing spots in adults.

To perform the test, larvae of three Drosophila strains (multiple wing hairs (*mwh/mwh*); *flare* (*flr3/In*(*3LR*) *TM3*, *ri p*^{p} *sep l*(*3*)*89Aa bx*^{34}*e e Bd*^{S}); and (iii) Oregon-*flare* (*ORR/ORR;flr3/ln*(*3LR*) *TM3*, *ri p*^{p} *sep l*(*3*)*89Aa bx4*^{3ec} *e Bd*^{S}) were fed with the material to be tested. The rapid cell proliferation (duplication time 10 h) renders the test sensitive. Gene mutations, chromosome breaks and rearrangements as well as mitotic recombinations lead to visible abnormalities allowing the detection of a wide range of genotoxins. Further advantages include low costs and minimal requirements for infrastructure. The Drosophila wing spot test has been used successfully for investigating the genotoxicity of NMs (Carmona et al., 2015a, 2015b, 2016; De Carli et al., 2018; Gorth et al., 2011; Machado et al., 2013; Reis et al., 2016; Vales et al., 2013). The experience with the use of the Drosophila wing spot assay for NM genotoxicity testing indicates that this test is suitable for investigating the potential genotoxicity of NMs.

12 | NEW ENDPOINTS OF NM GENOTOXICITY: DISTURBANCE OF GENE REGULATION AND EPIGENETICS

In several instances, it has been reported that exposure to NM has led to disturbed expression of genes involved in DNA damage response, DNA repair, or cell cycle regulation (Kohl et al., 2020; Rossner Jr et al., 2020; Smolkova et al., 2015) definitively or possibly leading to (secondary) genotoxicity (Smolkova et al., 2017). Changes in cell cycle-related gene expression have already been observed at much lower NM (cationic poly[amidoamine]dendrimers) doses than those required to lead to cytotoxicity in primary human bronchial epithelial cells (Feliu et al., 2016). NM-dependent changes in gene expression frequently involve many more than just one gene rather forming entire signal transduction pathways (Kim et al., 2019; Sima et al., 2020). In the mouse epidermal cell line JB6 P(+), Tungsten carbide–cobalt (WC–Co) NP activated both the transcription factors AP-1 and NF-kappa B and the mitogen-activated protein kinase (MAPK) signaling pathways much more efficiently than the fine WC–Co (AP-1 4-fold, MAPK 28-fold) (Lison et al., 2018).

Likewise, in several cases, exposure to NMs has been shown to lead to epigenetic changes, that is, to changes other than the DNA base sequence. Such changes include DNA methylations, histone modifications and changes in the occurrence of individual microRNAs (miRNAs, small noncoding, single-stranded RNAs). DNA methylations occur at the 5th position of cytosine within the partial DNA sequence CpG and govern the expression of individual genes at the transcriptional level. Histone modifications include acetylations, methylations, phosphorylations, and ubiquitinations governing the availability of DNA partial sequences for transcription. miRNAs substantially contribute to the regulation of gene expression at the translational level by controlling the stability of mRNA or their targeting for degradation. Such epigenetic changes as a consequence of exposure to NM have been observed repeatedly (Figure 1) (Smolkova et al., 2015, 2017, 2019; Stoccoro et al., 2013).

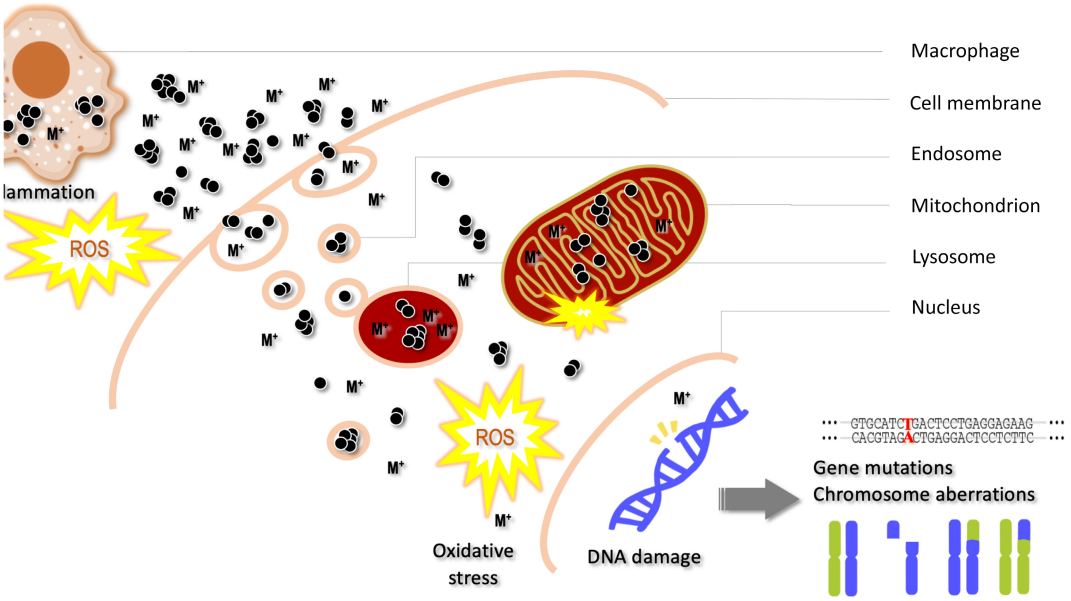

FIGURE 1 Overview of effects of nanoparticles potentially leading to mutations (gene mutations and chromosomal aberrations): Inflammatory processes, generation of reactive oxygen species (ROS), and release metal ions, potentially causing DNA damage

13 | CONCLUSIONS

Recent decades have undoubtedly brought substantial progress in the approaches to investigate the genotoxicity of NMs, as evidenced by the methods described in this review. Although no strict guidelines exist regarding a minimal battery of tests needed to satisfactorily ascertain safety from potential genotoxicities of NM, it is by now clear that gene mutations as well as chromosome/genome mutations should be considered. In addition, the preference among available tests to achieve this goal has gained a reasonable basis, as presented in detail in this review. In this respect, the use of mammalian cell gene mutation tests is preferred over bacterial gene mutation tests and the use of the MN test over chromosome aberration tests for chromosome mutations for the testing of NMs. The limitation of the comet assay also became apparent since the DNA damage seen in this test may be repaired before it is fixed as a mutation. In addition, some less frequently used tests for investigating NM genotoxicity have been discussed, such as the γ-H2AX test, which allows scoring toxicologically important DNA double-strand breaks, the pig-A test, which allows scoring of gene mutations in mammals in vivo, and the ToxTracker assay, which allows an investigation of the mechanisms underlying NM-induced genotoxicity.

The material collected and discussed in this review also showed some frequent shortcomings, such as insufficient characterization of the investigated NM. Some of the studies did not include a positive control; hence, in the case of a negative result, it was not possible to conclude whether the tested NM was devoid of the tested genotoxicity or the test did not function. Many studies use traditional positive control substances, which are small molecules, and there is a general lack of agreed positive control nanomaterials. In several studies, it was not clear whether negative results are due to the lack of genotoxicity of nanomaterials taken up by the cell or due to a lack of cellular uptake; obviously, both can substantiate the absence of genotoxicity. A major shortcoming is that adapted protocols were neither adapted nor validated for nanomaterial testing.

Many fundamentally important questions remain, but it is hoped that the facts collected and considerations put forward in this review help to approach more conclusive answers in the—hopefully not too distant—future. At present, all OECD test guidelines for genotoxicity assessments are—by default—applicable to nanomaterials. These guidelines were, however, developed and validated for small molecules. Currently, test guidelines are adapted and validated for nanomaterials; for this reason, the following considerations should be taken into account:

Relevance: Are we testing for genotoxicity leading to cancer (are there reference data on the mutagenic carcinogenicity of NM?) and/or germ cell mutagenicity? Which cell types and tissues are thus relevant (e.g., genotoxicity in lavaged lung cells which are nontarget cells)? What is then the relevant test system(s)? Are secondary genotoxic lesions to be considered, and how is this assessed concerning hazard and risk assessment (as this is a threshold mechanism with concomitant effects, e.g., inflammation).
Test systems: Which test systems are relevant (see above)? Are they adapted for NMs? Are they validated for NMs? (What would be the reference data? Which NMs would be agreed on as positive controls? Again, see above)? There is a lack of OECD test guidelines specifically adopted for NMs.
Dosing: Which concentrations should be tested? (see Keller et al., 2021; Ma-Hock et al., 2021). What would be a meaningful top concentration? Do the high doses interfere with the read-out or depletion of components by adsorption (Monteiro-Riviere et al., 2009; Wohlleben et al., 2011; Wörle-Knirsch et al., 2006)? How can the NM be introduced into the test system (test item preparation)? Which test systems are relevant because they allow cellular uptake, or is genotoxicity generally not relevant in the absence of cellular uptake? How can ion-mediated genotoxicity be differentiated from particle-mediated genotoxicity? (see Semisch et al., 2014; Strauch et al., 2017).
Overall testing strategy and prediction model: What is the recommended combination of test systems (testing battery and testing strategy, as discussed above)? How can mutagenicity be assessed based on multiple test results (knowing that the results of different tests can be discordant)? A priori definition of the applicability domains of individual tests/test combinations/test strategies? Is in vivo testing the ultima ratio or is a comprehensive in vitro testing strategy and/or grouping and read-across sufficient?

It is hoped that this review points out these fundamental questions and contributes a modest proportion to some answers, thereby helping to improve the genotoxicity testing and assessment of nanomaterials in the future.

AUTHOR CONTRIBUTIONS

Robert Landsiedel: Conceptualization (lead); funding acquisition (lead); resources (equal); supervision (equal); visualization (lead); writing – original draft (equal); writing – review and editing (lead). **Naveed Honarvar:** Data curation (equal); investigation (equal); methodology (equal); writing – review and editing (equal). **Svenja Berit Seiffert:** Investigation (supporting); methodology (supporting); writing – original draft (supporting). **Barbara Oesch:** Formal analysis (equal); investigation (equal); writing – review and editing (supporting). **Franz Oesch:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

Naveed Honarvar, Svenja Berit Seiffert and Robert Landsiedel are employees of BASF, a chemical company using nanotechnology.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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