We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

186,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS
BOOK CITATION INDEX
INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Progress in Genotoxicity Evaluation of Engineered Nanomaterials

Xiaoqing Guo and Tao Chen

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61013

Abstract

Engineered nanomaterials (ENMs) are being produced at an increasing rate. Because of their unique physicochemical properties, ENMs have been used in a wide variety of commercial products. The specific properties of ENMs, such as their relatively larger surface area, however, could also cause adverse biological effects different from their bulk counterparts. Nanomaterials can be genotoxic while their bulk counterparts are not, or vice versa, due to these specific characteristics. Also, the differences between nanomaterials and bulk materials can generate uncertainty when measuring the genotoxic potential of ENMs using current genotoxicity assays that were developed for conventional chemicals or bulk materials. In this chapter, we summarize current progress in evaluating the genotoxicity of ENMs with a focus on results from the standard genotoxicity assays, possible mechanisms underlying the genotoxicity of ENMs, the suitability of current genotoxicity assays for evaluation of ENMs, and application of ENM genotoxicity data for risk assessment. Future perspectives for the evaluation of ENM genotoxicity are also addressed.

Keywords: Engineered nanomaterial, genotoxicity, Ames test, Comet assay, Micronucleus assay, reactive oxygen species, risk assessment

1. Introduction

Nanomaterials (NMs) are generally defined as materials having at least one dimension ranging from 1 to 100 nm in size. They may exist in nature, or be purposely engineered from various materials, such as carbon or minerals. Materials engineered to nanoscale size are referred to

as engineered nanomaterials (ENMs). Recently, the European Commission Delegated Regulation redefined ENMs as "any intentionally manufactured material, containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm to 100 nm" [1]. ENMs can be categorized into four classes: carbon-based NMs such as carbon nanotubes and graphene, metal-based NMs such as quantum dots and metal or metal-oxide NMs, dendrimers such as nanosized polymers, and composite NMs [2].

Novel ENMs are now designed and produced at an increasing rate, and having unique physicochemical features such as small size, particular shapes, large surface areas, and surface activity. These features provide ENMs specific characteristics of high thermal and energy conductivity, durability, strength, and/or reactivity [3], which facilitate their applications in a whole host of areas ranging from aerospace, engineering, and nanoelectronics to medical healthcare. According to a Wilson Center study, more than 1,600 manufacturer-identified nanotechnology-based consumer products have been introduced to the market, and more than half were in the Health and Fitness subcategory [4]. A wide range of human-application-related ENM products have emerged in textiles, the food industry, cosmetics, sunscreens, and the biomedical field including gene/drug delivery platforms, biosensors, cell and tumor imaging, and cancer photothermal therapy [5, 6]. With their widespread human exposure, the potential health risks stemming from ENMs have drawn increasing attention since the first report highlighting the immediate need for evaluating possible adverse health, safety, and environmental impacts of ENMs published by the Royal Society and Royal Academy of Engineering in 2004 [7]. In the same year, a new scientific field named "Nanotoxicology" emerged to investigate the toxic effects of ENMs. The genotoxicity evaluation of ENMs has attracted much attention due to its importance for nanotechnology regulation and risk assessment [8].

Genotoxicity is the ability of substances to damage DNA, the genetic information, within organisms. Thus, genotoxic agents can give rise to mutations. Because mutations can lead to cancer, genotoxicity evaluation has been utilized widely to evaluate the carcinogenic potential of chemical and physical exposures. International organizations and regulatory agencies, such as the Organization for Economic Co-operation and Development (OECD) and the International Conference on Harmonization (ICH), have published consensus guidance documents that describe a battery of test assays for genotoxicity assessment to support regulatory decision-making. These *in vitro* and *in vivo* assays measure different genotoxicity endpoints such as DNA breaks, gene mutations, and chromosomal alterations [9, 10]. The most widely used genotoxicity battery includes the bacterial *Salmonella* mutagenicity test (the Ames test); *in vitro* mammalian cell assays, such as the Comet assay, the mouse lymphoma gene mutation assay (MLA), and the micronucleus (MN) assay; and *in vivo* assays, including the *in vivo* MN and Comet assays. Although designed for conventional chemicals and bulk materials, these assays have commonly been adopted for measuring the genotoxicity of ENMs. In this chapter, we summarize test results from the genotoxicity evaluation of ENMs, focusing on those using standard genotoxicity assays, possible mechanisms underlying the genotoxicity of ENMs, the suitability of current genotoxicity assays for the genotoxicity evaluation of ENMs, and the use of genotoxicity data for the risk assessment of ENMs.

2. Genotoxicity of ENMs

In comparison to the fast-growing ENM market, studies evaluating ENM genotoxicity are still limited [11]. A literature search performed by Magdolenova et al. [12] found that from 2000 to 2012 only 2.6% of articles on ENM toxicity describe genotoxicity studies. Although both positive and negative results have been reported on the genotoxicity of ENMs in various cell and animal test models, the existing data indicate that many ENMs are genotoxic [2, 11, 13, 14]. In order to provide a holistic picture of the current situation on genotoxicity testing of ENMs, we performed a literature search in PubMed using "nanoparticles" or "nanomaterial" and "Comet," "micronuclei," "Ames," "*Hprt*," or "mouse lymphoma assay" as key words. A total of 274 publications were identified; the distribution of year of publication is shown in Figure 1. There was a clear increase in the number of publications/year for both the *in vitro* and *in vivo* ENM genotoxicity studies, up to 2012, where a plateau may have been reached. Among the different assays used for evaluation of ENM genotoxicity in the publications, the Comet assay was the most frequently employed assay, followed by the MN assay, both *in vitro* (Figure 2A) and *in vivo* (Figure 2B). This observation is consistent with a previous report that summarized 112 ENM genotoxicity studies (94 *in vitro*, 22 *in vivo*) from years 2000 to 2012 [12].
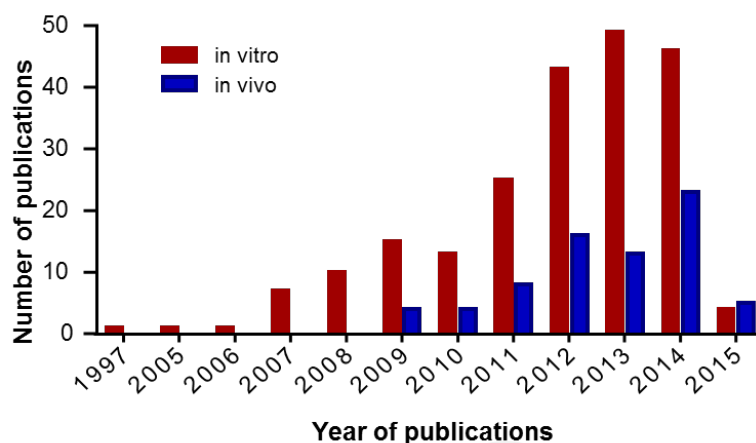

Figure 1. Literature review results of the publications per year on genotoxicity of engineered nanomaterials. Red bars indicate *in vitro* studies and blue bars indicate *in vivo* studies.

2.1. *In vitro* studies

A total of 215 publications were found that investigated the *in vitro* genotoxicity of ENMs. The Ames test is usually the initial step used for quickly screening potential mutagens and for identifying potential human carcinogens. This assay detects base substitution and frameshift mutations depending on the strain of the bacteria *Salmonella typhimurium* used [15]. Although this assay has been widely used for testing bulk materials, the Ames test was used less frequently for ENMs as compared to other genotoxicity assays [16]. Based on our literature search, among the most tested ENMs are nanoparticles (NPs) of silver (Ag), titanium dioxide (TiO$_2$), aluminium oxide (Al$_2$O$_3$), Zinc oxide, iron oxide, iron-platinum (FePt), single-wall
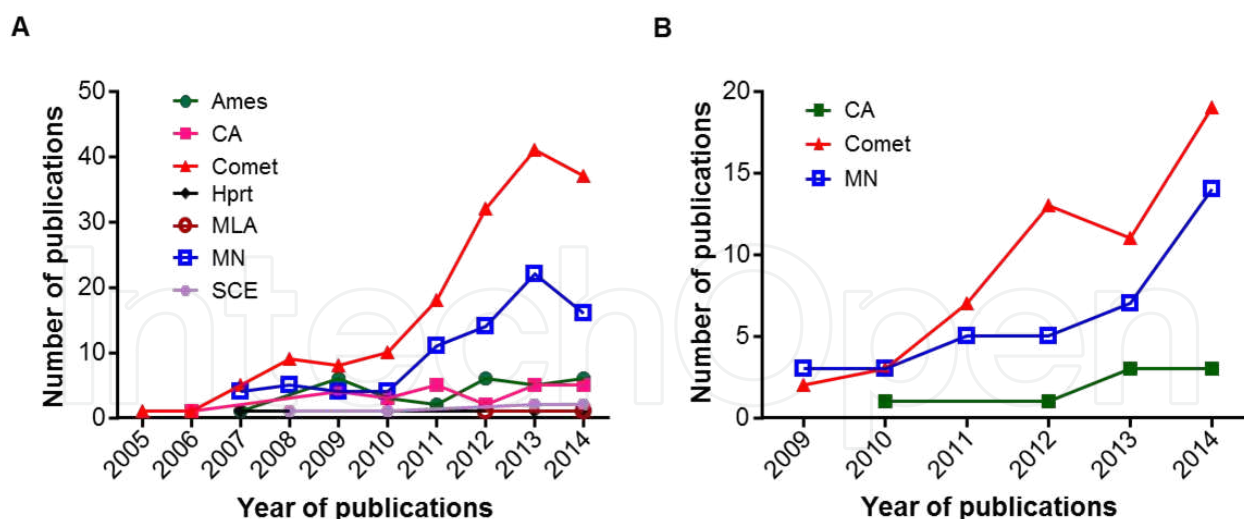
Figure 2. The trend in genotoxicity endpoint studies on engineered nanomaterials. (A) *in vitro* genotoxicity assays; (B) *in vivo* genotoxicity assays.

carbon nanotubes (SWCNTs), multiwalled carbon nanotubes (MWCNTs), and fullerenes [17-32]. Most of the Ames tests were negative both in the presence and the absence of metabolic activation. As shown in Table 1, only 27% of ENMs were positive in one or more tester strains in the Ames test. Most of the positive responses were weak or resulted from water-soluble NPs [16, 20, 24, 28, 30, 31]. Among possible explanations for the negative responses are the inability of the ENMs to penetrate the bacterial cell wall and the insensitivity of most of the tester strains to oxidative DNA damage, the primary mechanism for ENM genotoxicity [17]. In addition, the antimicrobial properties of some ENMs, such as Ag NPs, may limit the test concentrations due to cytotoxicity, thus reducing the sensitivity of the test [17]. In a study using 5 tester strains (TA98, TA100, TA1535, TA1537, and TA102) of *Salmonella typhimurium*, due to antimicrobial properties, the highest testable concentrations of 5 nm Ag NPs were 2-40 μg/plate, which is much lower than the limit of 5,000 μg/plate that is recommended for nontoxic test articles [17].

The Comet assay, also known as the single-cell gel electrophoresis assay, is a relatively simple and sensitive method for measuring DNA damage in individual eukaryotic cells [33]. Our literature review shows that 276 ENMs have been evaluated in 168 reports using the *in vitro* Comet assay. Thus, it is the most frequently used assay for ENM genotoxicity assessment (Table 1). The materials most tested with the Comet assay were TiO_2-, iron-, Ag-, and carbon-based ENMs. The majority of these tested ENMs (214 out of 276, 78%) induced DNA damage in various cell types in the standard or modified Comet assays. The high sensitivity of the assay may be ascribed to its ability to detect single- and double-strand breaks, cross-links, base damage, oxidative stress, DNA methylation, and apoptotic nuclei [33-35]. Some ENMs producing oxidative DNA damage were negative in the standard alkaline Comet assay, but were positive in the enzyme-modified Comet assay in which lesion-specific endonucleases were added to recognize particular oxidized nucleotides and create oxidized DNA-damage-specific breaks. The most commonly used modifying enzymes in these assays were formami-dopyrimidine DNA glycosylase (FPG), followed by endonuclease III (EndoIII) from *Escherichia*

coli and human-derived oxoguanine DNA glycosylase (hOGG1) [36]. Oxidative DNA lesions in FPG-sensitive sites were found in Fe_3O_4 NP-treated A549 type II lung epithelial cells [37], and in SWCNT- and C60-treated FE1-Muta™ Mouse lung epithelial cells [38].

Assays	Publications[b]	ENMs[c]	Positive outcomes[d]	Negative outcomes[d]
Comet	168	276	214 (78%)	62 (22%)
MN	83	126	75 (60%)	51 (40%)
Ames	30	55	15 (27%)	40 (73%)
CA	25	36	16 (44%)	20 (56%)
Hprt	6	8	5 (63%)	3 (37%)
SCE	6	9	6 (67%)	3 (33%)
MLA	2	2	2 (100%)	0 (0%)

[a]Data represent a literature review on the *in vitro* genotoxicity of engineered nanomaterials. Literature was obtained from the PubMed online database using "nanomaterial", "comet", "micronuclei", "ames", "*hprt*", or "mouse lymphoma assay" as key words. [b]The number of papers published using each assay. [c]The number of various ENMs tested in current literature citations. [d]The number of positive or negative outcomes for genotoxicity testing on ENMs. The number in parentheses indicates the percentage of positive or negative outcomes for each assay. CA, chromosome aberration; *Hprt*, hypoxanthine phosphorybosyl transferase assay; MLA, the mouse lymphoma assay; MN, the micronucleus assay; SCE, sister chromatid exchange.

Table 1. Summary of *in vitro* genotoxicity outcomes of engineered nanomaterials (ENMs)[a]

The MN assay detects chromosome fragments and whole chromosomes in the cytoplasm of interphase cells resulting from the clastogenic and aneugenic activities of mutagens, indicating chemical-induced chromosome damage (OECD TG487) [39]. The MN assay was the second most used assay for the evaluation of ENM genotoxicity (Table 1) [12, 16, 40]. Two versions of the MN assay, with or without pretreatment with cytochalasin B (cytoB) which blocks cytokinesis and results in binucleated cells, were used for NM studies [2, 39]. Approximately 60% of the tested ENMs produced concentration-dependent micronucleus formation or a positive response at high concentrations, including TiO_2 [8, 41], silicon dioxide (SiO_2) [42], cerium oxide (CeO_2) [43], cobalt-chromium (Co-Cr) [44, 45], MWCNTs [46, 47], and Ag NPs [17]. Kim et al. [29] demonstrated that in the absence of S9 metabolic activation, 10 μg/ml Ag NPs with an average size of 59 nm induced significantly greater MN formation using the protocol without cytoB than that with cytoB. It was demonstrated that cytoB could inhibit the cellular uptake of particulate materials and may contribute to negative results in MN assays [40].

The chromosome aberration (CA) test identifies agents that cause structural chromosome alterations in cultured mammalian cells (OECD TG473) [48]. This labor-intensive assay is routinely used for screening possible mammalian mutagens and carcinogens [14]. The CA assay was the third most commonly used mammalian cell assay for ENM genotoxicity investigation in our literature search (Table 1). Thirty-six ENMs including MWCNTs, C_{60}

fullerenes, Ag, and TiO$_2$ NPs have been evaluated using the CA test in 25 publications. Less than half (44%) of the tested ENMs were positive. Impressively, Ag NPs induced significant chromosomal aberrations at concentrations as low as 0.1 μg/ml in human mesenchymal stem cells [49] and at a concentration of 0.1 μg/cm^2 in a nonmammalian fish cell line [50].

Another assay used for detecting chromosome damage is the sister chromatid exchange (SCE) assay. This assay detects reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome [8]. Only 9 ENMs have been investigated using the SCE assay. The results revealed a surprisingly high positive response rate (67%) for the tested ENMs. SiO$_2$ NPs (at different sizes of 6, 20, 50 nm), which is considered relatively less genotoxic than other metal ENMs, significantly increased the SCE frequency in peripheral blood lymphocytes while they were negative in the MN assay [51].

The MLA and the hypoxanthine phosphorybosyl transferase (*Hprt*) assay, using thymidine kinase (*Tk*) and *Hprt* genes as target genes, respectively, are the most commonly used assays for the determination of chemical-induced gene mutations [52]. Both assays are included in the guidelines for mammalian gene mutation tests (OECD 476) [53]. There were only two MLA and six *Hprt* studies found in the literature. In the MLA studies, 5 nm uncoated Ag NPs produced dose-dependent cytotoxicity and mutagenicity at doses of 3-6 μg/ml [34], and tungsten carbide-cobalt (WC-Co) NPs with a diameter of 20-160 nm induced significant increases in cytotoxicity and mutagenicity following both 4 h and 24 h treatments [54]. Six *Hprt* studies investigated the mutagenic effect of four ENMs, TiO$_2$, SiO$_2$, ultrafine quartz, and SWCNTs, with positive results for *Hprt* gene mutation found for TiO$_2$, SiO$_2$, and ultrafine quartz [41, 42, 55-58]. Manshian et al. [58] investigated the genotoxicity of three sizes of SWCNTs, with a diameter of 1-2 nm and a length of 400-800 nm, 1-3 μm, or 5-30 μm, using MCL-5 human B-lymphoblastoid cells. Only the 1-3 μm SWCNTs significantly increased *Hprt* point mutations at concentrations ≥25 μg/ml. A chronic exposure of Chinese hamster ovary (CHO-K1) cells with TiO$_2$ NPs at up to 40 μg/ml for 60 days produced negative results [57].

2.2. *In vivo* studies

In vivo responses reflect the systematic biodistribution of ENMs and evaluate the cytotoxicity/genotoxicity to different tissues and organs. Our literature search identified 73 publications on the *in vivo* genotoxicity of ENMs, with the number of publications increasing with time (Figure 1). As shown in Table 2, the Comet assay remains the most frequently used *in vivo* assay for investigating ENM genotoxicity, followed by the *in vivo* MN assay and then the CA assay. Both the *in vivo* MN and Comet assays are recommended by OECD (OECD TG 474 and 489) and ICH for regulatory decision-making [10, 59, 60]. Some ENMs, such as MWCNTs [47], carbon black [61], TiO$_2$ [8, 56, 62], CdSe quantum dots [63], and Ag NPs [64, 65], caused both DNA strand breaks and chromosomal damage in experimental animals (mainly mice and rats). However, in contrast to the high proportion of positive outcomes in *in vitro* studies, about half of the *in vivo* MN and Comet assay studies were negative for the tested ENMs (Table 2), probably due to the higher DNA repair capacity inherent to *in vivo* models as compared to *in vitro* models. It is worth noting that some of the positive ENMs were genotoxic only at the highest doses tested or in enzymatically modified Comet assays. In the liver of male B6C3F1

mice intravenously administered 15-100 nm PVP or 10-80 nm silicon-coated Ag NPs at a dose of 25 mg/kg/day for 3 consecutive days, no increase in DNA breaks was observed with the standard Comet assay. However, significant induction of oxidative DNA damage by the Ag NP treatment was detected with the EndoIII and hOOG1-modified Comet assay in the B6C3F1 mouse livers [65]. Interestingly, in the 8 publications using the *in vivo* CA assay, 75% of test articles were positive, which was much higher than the positive responses (44%) detected with *in vitro* CA assay testing of ENMs. This finding may be ascribed to the limited numbers of ENMs tested in the *in vivo* studies. The ENMs that did increase CA frequencies were CeO_2 [66, 67], TiO_2 [68], Ag [69], and MnO_2 NPs [70, 71]. Similar to the *in vitro* genotoxicity studies, inconsistent genotoxic outcomes were reported with *in vivo* models as well; however, there remains convincing evidence that ENMs can be genotoxic *in vivo*, depending on particle size, surface coating, exposure route, and exposure duration [72].

Assays	Publications[b]	ENMs[c]	Positive outcomes[d]	Negative outcomes[d]
Comet	60	87	49 (56%)	38 (44%)
MN	38	60	28 (47%)	32 (53%)
CA	8	8	6 (75%)	2 (25%)

[a-d] See Table 1 for notes.

Table 2. Summary of *in vivo* genotoxicity outcomes of engineered nanomaterials (ENMs)[a]

3. Possible mechanisms underlying genotoxicity of ENMs

ENMs are engineered to possess unique physicochemical properties that may have the potential to induce genotoxicity through different mechanisms [14]. Although these genotoxic mechanisms are still uncertain at present, studies indicate that ENMs can induce genotoxicity both directly and indirectly. Among these mechanisms, ENM-induced genotoxicity is most often attributed to oxidative stress.

3.1. Oxidative stress

A large number of studies suggest that oxidative stress plays a key role in ENM-induced genotoxicity [73, 74]. Induction of oxidative stress from ENM exposures could be the result of increased reactive oxygen species (ROS) or depletion of antioxidant defense molecules because of the high surface area of ENMs and their interaction with cells and cellular components [14, 73]. Increased ROS can modify DNA bases to induce oxidative DNA adducts, DNA single- and double-strand breaks, DNA cross-links, and DNA-protein cross-links [14, 75]. If ENM-induced DNA damage is not repaired, these DNA modifications and lesions can potentially cause mutations [76]. The correlation between oxidative stress and ENM-induced genotoxicity has been well documented in a number of studies using the Comet assay, γ-H2AX assay, and 8-hydroxy-2′-deoxyguanosine (8-OH-dG) assay [77].

To determine whether the mutagenicity of Ag NPs resulted from an oxidative stress mechanism [34], we conducted the standard Comet assay concurrent with the oxidative stress Comet assay. In the oxidative stress Comet assay, addition of FPG, EndoIII and hOGG1 lesion-specific endonucleases produces secondary DNA breaks by cutting at oxidative DNA adducts. While the Ag NP treatment of the cells did not increase DNA breaks in the standard assay, the FPG, EndoIII and hOGG1 modified Comet assays did detect oxidative DNA damages (Figure 3). These results suggest that Ag NP exposures induced oxidized nucleotides that could result in mutations.
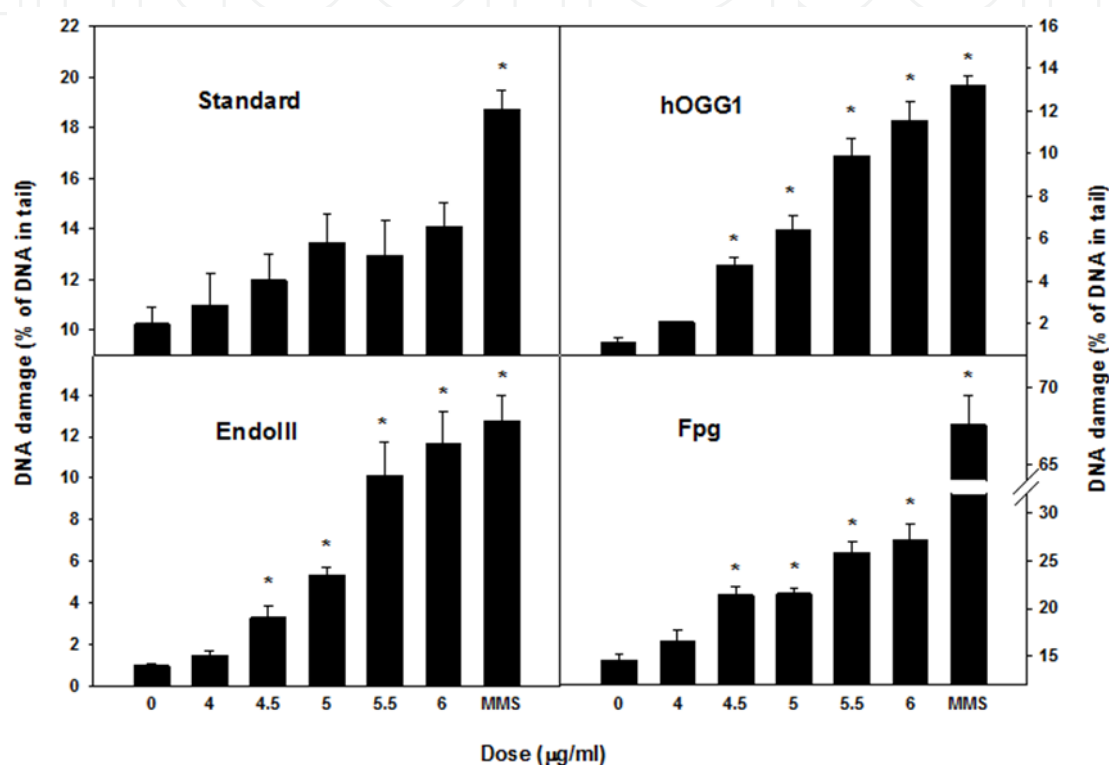

Figure 3. Silver nanoparticles induce DNA damage via oxidative stress. Mouse lymphoma cells were treated with 5nm Ag NPs for 4 hours. DNA damage was measured by the Comet assay. In the standard Comet assay, DNA breaks increased insignificantly with the increasing concentrations. In the oxidative stress Comet assay, however, addition of the different lesion-specific endonucleases (hOGG1, EndoIII, and Fpg) that cut different oxidative DNA adducts resulted in clear concentrations-dependent increases in DNA breaks. * indicates $p < 0.01$ when compared with the control group.

3.2. Direct interaction with DNA and nuclear protein

Due to their small size and charged surfaces, ENMs may be internalized through cellular membranes, reach the nucleus by diffusion across the nuclear membrane or by penetrating during mitosis. Thus, they can directly interact with DNA. During interphase, ENMs can chemically bind to DNA molecules and influence DNA replication that could result in DNA damage. It has been shown that NPs can dissociate double-stranded DNA [78] and cause clastogenic effects (breaks in chromosomes) or aneugenic effects (producing abnormal number of chromosomes) [12].

ENMs also can directly bind to DNA-related proteins leading to intranuclear protein aggregates that inhibit DNA replication, transcription, cell proliferation, and DNA repair [12, 14, 79]. It has been reported that SiO_2 NPs can enter the cell nucleus and cause aberrant clusters of topoisomerase I in the nucleoplasm [79]. *In silico* studies have identified a binding site for fullerene ENMs, on human DNA topoisomerase II alpha [80], and potential interactions between ENMs and proteins involved in the DNA mismatch repair pathway [81].

3.3. Ions released from ENM surfaces

Toxic metal ions can easily release from ENMs into their surrounding environment due to their relatively large surface area. These ions may exert genotoxic effects by the production of intracellular ROS [12], by binding to cellular macromolecules, or by activating mitogenic signaling pathways and inducing the expression of cellular proto-oncogenes [82]. For example, Co NPs can release Co^{2+} ions into the culture media [83], and Co^{2+} is a topoisomerase II poison that stimulates DNA cleavage in human MCF-7 cells [84] and induces micronuclei in the MN assay [85].

3.4. Inflammation

Inflammation is an important protective defense against tissue injury and infection. However, it can also induce genotoxicity in the form of DNA single- and double-strand breaks, chromosome fragmentation, point mutations, and DNA repair deficiency [14, 86]. Silica NP exposures induce genotoxic effects in male Wistar rats through an inflammatory reaction [87]. Many other ENMs, such as TiO_2, carbon black, magnetite iron, CeO_2, SWCNTs, and MWCNTs, can generate various degrees of inflammatory reactions, including increased expression of pro-inflammatory cytokines and inflammation-related genes, and formation of microgranulomas in treated animals [88, 89]. In addition, the association between ROS and inflammation has been demonstrated, where generation of inflammation and activated inflammatory cells can increase ROS production [90].

Chronic rat inhalation studies showed that TiO_2 NPs caused bronchoalveolar adenomas and cystic keratinizing squamous cell carcinomas, as well as alveolar/bronchiolar adenoma. Generation of ROS and induction of inflammation by TiO_2 NPs resulting in oxidative stress and genotoxicity were considered important factors in the initiation and progression stages of TiO_2 NP carcinogenesis [8, 91].

4. Approaches to the risk assessment of ENM genotoxicity

4.1. Suitability of current genotoxicity assays for evaluation of ENMs

A complete genotoxicity evaluation of a test agent required by regulatory agencies generally involves using a test battery that includes a bacterial gene mutation assay (e.g., the Ames test), an *in vitro* cytogenetic assay in mammalian cells and/or the mouse lymphoma mutation assay, and an *in vivo* cytogenetic assay (e.g., the *in vivo* rodent MN assay). However, these assays were developed for the evaluation of conventional chemicals and bulk materials. Whether or

not they are suitable for measuring ENM genotoxicity is still under investigation. ENM genotoxicity modes of action are not very clear and not always predictable. Thus, it remains a question as to whether the standard tests are appropriate and if they are sufficient.

Because bacterial mutation analysis is sometimes the only assay used for genotoxicity testing, it is very important to assess the utility of the Ames test for evaluating ENM mutagenicity. Most of the Ames test data for ENMs thus far reported are negative or only very weakly positive due to the inability of many ENMs to penetrate through the bacterial cell wall and the antimicrobial activity of some ENMs [16]. Our previous study indicated that TiO_2 NPs are not able to penetrate the cell wall of *Salmonella* tester strains [18]. Thus, there is a growing concern as to whether the Ames test is appropriate for evaluating ENM genotoxicity [92]. Since the Ames test is an important assay for measuring mutagenicity, if it is excluded from the genotoxicity test battery for ENMs, it may be necessary to include a mammalian cell gene mutation assay in the test battery [92].

Other standard genotoxicity assays used for evaluating NMs have shown inconsistent results. The most sensitive method for measuring ENM genotoxicity is the Comet assay. This assay produces the greatest number of positive results for ENMs both *in vitro* and *in vivo* (Table 1 and 2). The MN assay is another commonly used assay for detecting chromosome damage caused by ENMs. The MN data reveal that the *in vitro* assay is more sensitive than the *in vivo* assay. Mammalian gene mutation assays (*Hprt*, MLA, and transgenic mutation assays) and the CA assay are less frequently used than the Comet and MN assays. These genotoxicity assays are generally accepted for evaluation of ENMs with certain modifications. CytoB interferes with the uptake of ENMs and thus the binucleated MN assay should be used with caution. Also, the potential lack of uptake of agglomerated NMs by cells, and possible interference of NMs with endpoint measurement when measuring fluorescence should be considered, since these factors create a false positive or false negative result [92].

4.2. Regulatory approaches

For more than 40 years, the OECD has played an important regulatory role in ensuring the safe use of chemicals. In response to the fast-growing commercial applications for ENMs, the OECD established a project entitled "Manufactured Nanomaterials and Test Guidelines" in 2006 to ensure that the risk assessment of ENMs would be conducted in a suitable, science-based and internationally harmonized manner. After six years of work, the OECD concluded that it was unnecessary to develop completely new testing approaches for NMs and that most current test guidelines for assessment of traditional chemicals were in general applicable for ENMs. However, it was indicated that in some cases, modifications were needed to adapt current test guidelines for ENM specifications [93]. The OECD recently published a report on the genotoxicity testing of ENMs, in which some consensus statements were addressed [94]. Major recommendations were that the Ames test is not recommended for investigating the genotoxicity of ENMs; ENM characterization should be undertaken in the cell culture medium both at the beginning and after the treatment; the extent of cellular uptake is a critical factor to consider and cell lines that can take up ENMs are preferred for genotoxicity testing; cytoB should be added only postexposure or using a delayed cotreatment protocol for the *in vitro*

binucleated MN assay; pharmacokinetic investigations need to be conducted to determine if the ENMs reach the target tissue for *in vivo* studies; and the route most applicable to human exposure should be selected for ENM genotoxicity testing.

The U.S. Food and Drug Administration (FDA) has also made significant efforts to address the regulatory issues surrounding ENM products. The FDA Nanotechnology Task Force report addressed scientific and regulatory issues regarding to the safety and effectiveness of FDA-regulated products containing NMs. Also, the FDA issued a draft document on the use of nanotechnology in food for animals and three final guidance documents in 2014 related to nanotechnology application in regulated products, including cosmetics and food substances [95]. These documents assert that *in vitro* and *in vivo* mutagenicity/genotoxicity data are considered as one of the important factors for the safety assessment of ENMs. These guidance documents mainly focus on ENMs because materials manipulated on the nanoscale level may have altered biocompatibility and/or toxicity [96]. For example, some tea polyphenols in their bulk form have opposite effects on DNA damage in treated cells to those in their nano form [97].

4.3. Proposed tiered approaches for genotoxicity testing

Despite some discrepancies in the current literature, there is compelling evidence that some ENMs are genotoxic and potentially carcinogenic in living systems. Thus, multidisciplinary tiered toxicity testing approaches using different models and test methods are proposed for risk assessment of various ENMs [11, 98]. These proposals emphasize that a thorough characterization of the physicochemical properties of ENMs should be the first step in the evaluation of their genotoxicity. Then an *in silico* study should follow to simulate quantitative structure activity relationships between ENMs and their potential interaction with cellular macromolecules such as DNA and protein [98]. If positive, *in vitro* studies should be performed to assess the dose-response effects on cytotoxicity and genotoxicity of target cells, followed by mechanistic studies and *in vivo* studies to validate *in vitro* results. Positive outcomes for genotoxicity and mutagenicity may suggest a need for conducting further carcinogenicity and reproductive toxicity tests [11]. The proposed tiered approaches remain to be validated in the future.

5. Future perspectives

According to the current literature addressing ENM genotoxicity, a lack of physicochemical characterization, especially characterizing the ENMs in the testing medium, remains the biggest problem in most studies, and this may account for some of the conflicting test results. Inconsistency in dose metrics and the test systems also are important factors affecting the comparisons between studies [14]. Thus, the following should be taken into account for improving the genotoxicity evaluation of ENMs.

1. Comprehensively detailed physicochemical characterization of ENMs should be performed before and during any genotoxicity study. Important properties that can influence ENM-induced genotoxicity include size, coating, shape, chemical composition, crystal

structure, purity, surface area, surface chemistry, surface charge, solubility, and agglomeration. Measurements should include the stability of ENMs in the relevant test medium, such as aggregation status and ion release from metallic ENMs.

2. A battery of standard genotoxicity assays covering a wide range of mechanisms specifically tailored for ENMs is needed, since current studies generally use genotoxicity test batteries adapted for traditional chemicals. A mammalian mutation assay such as the MLA or an *Hprt* assay should be used in the battery given that the Ames test is not appropriate for testing the mutagenicity of ENMs. Integration of cytotoxicity assessment into genotoxicity evaluations will aid in avoiding false-positive results, especially in situations where genotoxicity can only be observed at excessively high concentrations with high cytotoxicity.

3. Appropriate controls, specifically ENM-related positive controls, need to be included in ENM genotoxicity tests. NMs may be different from their bulk materials in terms of uptake, absorption, transport into cells, and transport across barriers (e.g., blood-brain barrier) or have altered bioavailability or biological half-life [99]. Positive controls from bulk materials may or may not have the same tissue distributions as the corresponding nanoscale materials. If a soluble bulk material used as a positive control for a genotoxicity assay can reach a specific tissue for testing while insoluble ENMs cannot get into the target tissue, a false negative result could occur. Thus, ENM-related positive controls will benefit the genotoxicity evaluation of ENMs, and eventually ENM hazard identification and risk assessment.

4. Long-term impacts of ENM exposures need to be considered. To date, only a few studies are available describing the chronic and subchronic toxicity of ENMs *in vivo* or *in vitro*. Although no cytotoxicity or genotoxicity were observed for a chronic exposure of TiO_2 in Chinese hamster ovary cells [57], carbon-based ENMs could be visualized in the lung of mice following a 1-year pulmonary exposure, and SWCNT exposures increased the rate of K-*ras* mutations, MN formation, and nuclear protrusions in pulmonary epithelial cells [100].

5. Adopting high-throughput approaches, i.e., CometChip technology, for detection of DNA damage will facilitate screening a large number of ENMs. In addition, other endpoints and biomarkers may need to be considered for ENM genotoxicity assessment since a variety of molecular pathways, autophagy, and epigenetic alterations have been reported to be involved in ENM-induced genotoxic effects [101-105].

Acknowledgements

Author details

Xiaoqing Guo* and Tao Chen*

*Address all correspondence to: xiaoqing.guo@fda.hhs.gov; tao.chen@fda.hhs.gov

Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, USA

References

[1] EU Commissions Delegated Regulation, No 1363/2013 amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council on the provision of food information to consumers as regards the definition of 'engineered nanomaterials,' in *Off J EU*. 2013. p. L 343/26-28.

[2] Ng, C.T., et al. Current studies into the genotoxic effects of nanomaterials. *J Nucleic Acids*, 2010. 947859.

[3] Powell, M.C. and M.S. Kanarek. Nanomaterial health effects--part 1: background and current knowledge. *WMJ*, 2006. 105(2): p. 16-20.

[4] Wilson Center. Consumer Products Inventory. The project on emerging nanotechnologies. http://www.nanotechproject.org/cpi/about/analysis/, 2015.

[5] Ahamed, M., M.S. Alsalhi, and M.K. Siddiqui. Silver nanoparticle applications and human health. *Clin Chim Acta*, 2010. 411(23-24): p. 1841-8.

[6] Guo, X. and N. Mei. Assessment of the toxic potential of graphene family nanomaterials. *J Food Drug Anal*, 2014. 22(1): p. 105-15.

[7] Royal Society & The Royal Academy of Engineering report. *Nanoscience and nanotechnologies: opportunities and uncertainties.* http://www.nanotec.org.uk/report/Nano%20report%202004%20fin.pdf, 2004: p. 1-127.

[8] Chen, T., J. Yan, and Y. Li. Genotoxicity of titanium dioxide nanoparticles. *J Food Drug Anal*, 2014. 22(1): p. 95-104.

[9] FDA. *Toxicological Principles for the Safety Assessment of Food Ingredients*. Redbook 2000: IV.C.1 Short-Term Tests for Genetic Toxicity 2007 [cited 2014 september 16].

[10] ICH, *Guidance for Industry S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use.* http://www.fda.gov/downloads/Drugs/Guidances/ucm074931.pdf, 2012: p. 1-28.

[11] Savolainen, K., et al. Risk assessment of engineered nanomaterials and nanotechnologies--a review. *Toxicology*, 2010. 269(2-3): p. 92-104.

[12] Magdolenova, Z., et al. Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. *Nanotoxicology*, 2014. 8(3): p. 233-78.

[13] Magaye, R., et al. Genotoxicity and carcinogenicity of cobalt-, nickel- and copper-based nanoparticles. *Exp Ther Med*, 2012. 4(4): p. 551-61.

[14] Singh, N., et al. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. *Biomaterials*, 2009. 30(23-24): p. 3891-914.

[15] Claxton, L.D., et al. Guide for the Salmonella typhimurium/mammalian microsome tests for bacterial mutagenicity. *Mutat Res*, 1987. 189(2): p. 83-91.

[16] Landsiedel, R., et al. Genotoxity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations–many questions, some answers. *Mutat Res*, 2009. 681(2-3): p. 241-58.

[17] Li, Y., et al. Genotoxicity of silver nanoparticles evaluated using the Ames test and in vitro micronucleus assay. *Mutat Res*, 2012. 745(1-2): p. 4-10.

[18] Woodruff, R.S., et al. Genotoxicity evaluation of titanium dioxide nanoparticles using the Ames test and Comet assay. *J Appl Toxicol*, 2012. 32(11): p. 934-43.

[19] Di Sotto, A., et al. Multi-walled carbon nanotubes: Lack of mutagenic activity in the bacterial reverse mutation assay. *Toxicol Lett*, 2009. 184(3): p. 192-7.

[20] Gomaa, I.O., et al. Evaluation of in vitro mutagenicity and genotoxicity of magnetite nanoparticles. *Drug Discov Ther*, 2013. 7(3): p. 116-23.

[21] Han, D.W., et al. In-vivo and in-vitro biocompatibility evaluations of silver nanoparticles with antimicrobial activity. *J Nanosci Nanotechnol*, 2012. 12(7): p. 5205-9.

[22] Hu, P., et al. Genotoxicity evaluation of stearic acid grafted chitosan oligosaccharide nanomicelles. *Mutat Res*, 2013. 751(2): p. 116-26.

[23] Landsiedel, R., et al. Gene toxicity studies on titanium dioxide and zinc oxide nanomaterials used for UV-protection in cosmetic formulations. *Nanotoxicology*, 2010. 4: p. 364-81.

[24] Maenosono, S., Suzuki, T., and Saita, S. *Mutagenicity of water-soluble FePt nanoparticles in Ames test. J Toxicol Sci*, 2007. 32(5): p. 575-9.

[25] Naya, M., et al. Evaluation of the genotoxic potential of single-wall carbon nanotubes by using a battery of in vitro and in vivo genotoxicity assays. *Regul Toxicol Pharmacol*, 2011. 61(2): p. 192-8.

[26] Shinohara, N., et al. In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles. *Toxicol Lett*, 2009. 191(2-3): p. 289-96.

[27] Wirnitzer, U., et al. Studies on the in vitro genotoxicity of baytubes, agglomerates of engineered multi-walled carbon-nanotubes (MWCNT). *Toxicol Lett*, 2009. 186(3): p. 160-5.

[28] Hasegawa, G., Shimonaka, M., and Ishihara, Y. Differential genotoxicity of chemical properties and particle size of rare metal and metal oxide nanoparticles. *J Appl Toxicol*, 2012. 32(1): p. 72-80.

[29] Kim, H.R., et al. Appropriate in vitro methods for genotoxicity testing of silver nanoparticles. *Environ Health Toxicol*, 2013. 28: p. e2013003.

[30] Liu, Y., et al. Genotoxicity assessment of magnetic iron oxide nanoparticles with different particle sizes and surface coatings. *Nanotechnology*, 2014. 25(42): p. 425101.

[31] Jomini, S., et al. Modifications of the bacterial reverse mutation test reveals mutagenicity of TiO(2) nanoparticles and byproducts from a sunscreen TiO(2)-based nanocomposite. *Toxicol Lett*, 2012. 215(1): p. 54-61.

[32] Balasubramanyam, A., et al. In vitro mutagenicity assessment of aluminium oxide nanomaterials using the Salmonella/microsome assay. *Toxicol In Vitro*, 2010. 24(6): p. 1871-6.

[33] Tice, R.R., et al. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen*, 2000. 35(3): p. 206-21.

[34] Mei, N., et al. Silver nanoparticle-induced mutations and oxidative stress in mouse lymphoma cells. *Environ Mol Mutagen*, 2012. 53(6): p. 409-419.

[35] Olive, P.L., and Banath J. P. The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc*, 2006. 1(1): p. 23-9.

[36] Moller, P., et al. Applications of the comet assay in particle toxicology: air pollution and engineered nanomaterials exposure. *Mutagenesis*, 2015. 30(1): p. 67-83.

[37] Karlsson, H.L., et al. Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. *Chem Res Toxicol*, 2008. 21(9): p. 1726-32.

[38] Jacobsen, N.R., et al. Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C(60) fullerenes in the FE1-Mutatrade mark-Mouse lung epithelial cells. *Environ Mol Mutagen*, 2008. 49(6): p. 476-87.

[39] OECD. *In Vitro Mammalian Cell Micronucleus Test, OECD Guideline for Testing of Chemicals, No. 487.* http://www.oecd-ilibrary.org/docserver/download/9714561e.pdf?expires=1417033814&id=id&accname=guest&checksum=A77BB1B023EACFEF8C36FD865B6981D3, 2014.

[40] Oesch, F., and Landsiedel, R. Genotoxicity investigations on nanomaterials. *Arch Toxicol*, 2012. 86(7): p. 985-94.

[41] Wang, J.J., B.J. Sanderson, and Wang, H. Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells. *Mutat Res*, 2007. 628(2): p. 99-106.

[42] Wang, J.J., B.J. Sanderson, and Wang, H. Cytotoxicity and genotoxicity of ultrafine crystalline SiO2 particulate in cultured human lymphoblastoid cells. *Environ Mol Mutagen*, 2007. 48(2): p. 151-7.

[43] Benameur, L., et al. DNA damage and oxidative stress induced by CeO nanoparticles in human dermal fibroblasts: Evidence of a clastogenic effect as a mechanism of genotoxicity. *Nanotoxicology*, 2014: p. 1-10.

[44] Papageorgiou, I., et al. The effect of nano- and micron-sized particles of cobalt-chromium alloy on human fibroblasts in vitro. *Biomaterials*, 2007. 28(19): p. 2946-58.

[45] Tsaousi, A., Jones, E., and Case, C.P. The in vitro genotoxicity of orthopaedic ceramic (Al2O3) and metal (CoCr alloy) particles. *Mutat Res*, 2010. 697(1-2): p. 1-9.

[46] Kato, T., et al. Genotoxicity of multi-walled carbon nanotubes in both in vitro and in vivo assay systems. *Nanotoxicology*, 2013. 7(4): p. 452-61.

[47] Muller, J., et al. Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. *Carcinogenesis*, 2008. 29(2): p. 427-33.

[48] OECD. *In Vitro Mammalian Chromosome Aberration Test*. OECD Guideline for the Testing of Chemicals: TG473, 2014. http://www.oecd-ilibrary.org/docserver/download/9714531e.pdf?expires=1417033602&id=id&accname=guest&checksum=232CA39FC18225209745E6BAE3AFADB4.

[49] Hackenberg, S., et al. Silver nanoparticles: evaluation of DNA damage, toxicity and functional impairment in human mesenchymal stem cells. *Toxicol Lett*, 2011. 201(1): p. 27-33.

[50] Wise, J.P., Sr., et al. Silver nanospheres are cytotoxic and genotoxic to fish cells. *Aquat Toxicol*, 2010. 97(1): p. 34-41.

[51] Battal, D., et al. SiO Nanoparticule-induced size-dependent genotoxicity - an in vitro study using sister chromatid exchange, micronucleus and comet assay. *Drug Chem Toxicol*, 2014: p. 1-9.

[52] Johnson, G.E. Mammalian cell HPRT gene mutation assay: test methods. *Methods Mol Biol*, 2012. 817: p. 55-67.

[53] OECD. *In Vitro Mammalian Cell Gene Mutation Test*. OECD Guideline for the Testing of Chemicals, 1997. http://www.oecd.org/chemicalsafety/risk-assessment/1948426.pdf.

[54] Moche, H., et al. Tungsten carbide-cobalt as a nanoparticulate reference positive control in in vitro genotoxicity assays. *Toxicol Sci*, 2014. 137(1): p. 125-34.

[55] Wang, J.J., Wang, H., and Sanderson, B.J. Ultrafine Quartz-Induced Damage in Human Lymphoblastoid Cells in vitro Using Three Genetic Damage End-Points. *Toxicol Mech Methods*, 2007. 17(4): p. 223-32.

[56] Chen, Z., et al. Genotoxic evaluation of titanium dioxide nanoparticles in vivo and in vitro. *Toxicol Lett*, 2014. 226(3): p. 314-19.

[57] Wang, S., et al. Chronic exposure to nanosized, anatase titanium dioxide is not cyto- or genotoxic to Chinese hamster ovary cells. *Environ Mol Mutagen*, 2011. 52(8): p. 614-22.

[58] Manshian, B.B., et al. Single-walled carbon nanotubes: differential genotoxic potential associated with physico-chemical properties. *Nanotoxicology*, 2013. 7(2): p. 144-56.

[59] OECD. *In Vivo Mammalian Alkaline Comet assay.* OECD Guideline for Testing of Chemicals, No. 489, 2014. http://www.oecd-ilibrary.org/docserver/download/ 9714511e.pdf?expires=1417034151&id=id&accname=guest&check- sum=B65AE67EDF756C95A5EC39B3A518DB0B.

[60] OECD. *Mammalian Erythrocyte Micronucleus Test.* OECD Guideline for THE Testing of Chemicals, No. 474, 2014. http://www.oecd-ilibrary.org/docserver/download/ 9714541e.pdf?expires=1417034451&id=id&accname=guest&check- sum=8CAB5C07D1DEDD0CB07A7B9A3662157A.

[61] Bourdon, J.A., et al. Carbon black nanoparticle instillation induces sustained inflam- mation and genotoxicity in mouse lung and liver. *Part Fibre Toxicol*, 2012. 9: p. 5.

[62] El-Ghor, A.A., et al. Normalization of Nano-Sized TiO2-Induced Clastogenicity, Gen- otoxicity and Mutagenicity by Chlorophyllin Administration in Mice Brain, Liver, and Bone Marrow Cells. *Toxicol Sci*, 2014. 142(1): p. 21-32.

[63] Khalil, W.K., et al. Genotoxicity evaluation of nanomaterials: dna damage, micronu- clei, and 8-hydroxy-2-deoxyguanosine induced by magnetic doped CdSe quantum dots in male mice. *Chem Res Toxicol*, 2011. 24(5): p. 640-50.

[64] Dobrzynska, M.M., et al. Genotoxicity of silver and titanium dioxide nanoparticles in bone marrow cells of rats in vivo. *Toxicology*, 2014. 315: p. 86-91.

[65] Li, Y., et al. Cytotoxicity and genotoxicity assessment of silver nanoparticles in mouse. *Nanotoxicology*, 2014. Suppl 1: p. 36-45.

[66] Kumari, M., Kumari, S.I., and Grover, P. Genotoxicity analysis of cerium oxide micro and nanoparticles in Wistar rats after 28 days of repeated oral administration. *Muta- genesis*, 2014. 29(6): p. 467-79.

[67] Kumari, M., et al. Genotoxicity assessment of cerium oxide nanoparticles in female Wistar rats after acute oral exposure. *Mutat Res Genet Toxicol Environ Mutagen*, 2014. 775-776: p. 7-19.

[68] Pakrashi, S., et al. In vivo genotoxicity assessment of titanium dioxide nanoparticles by Allium cepa root tip assay at high exposure concentrations. *PLoS One*, 2014. 9(2): p. e87789.

[69]   Ghosh, M., et al. In vitro and in vivo genotoxicity of silver nanoparticles. *Mutat Res*, 2012. 749(1-2): p. 60-9.

[70]   Singh, S.P., et al. Genotoxicity of nano- and micron-sized manganese oxide in rats after acute oral treatment. *Mutat Res*, 2013. 754(1-2): p. 39-50.

[71]   Singh, S.P., et al. Toxicity assessment of manganese oxide micro and nanoparticles in Wistar rats after 28 days of repeated oral exposure. *J Appl Toxicol*, 2013. 33(10): p. 1165-79.

[72]   Klien, K., and Godnic-Cvar, J. Genotoxicity of metal nanoparticles: focus on in vivo studies. *Arh Hig Rada Toksikol*, 2012. 63(2): p. 133-45.

[73]   Kermanizadeh, A., et al. The role of intracellular redox imbalance in nanomaterial induced cellular damage and genotoxicity: A review. *Environ Mol Mutagen*, 2015. 56(2):p. 111-24.

[74]   Fu, P.P., et al. Mechanisms of nanotoxicity: generation of reactive oxygen species. *J Food Drug Anal*, 2014. 22(1): p. 64-75.

[75]   Schieber, M., and Chandel, N.S. ROS function in redox signaling and oxidative stress. *Curr Biol*, 2014. 24(10): p. R453-62.

[76]   Toyokuni, S. Oxidative stress and cancer: the role of redox regulation. *Biotherapy*, 1998. 11(2-3): p. 147-54.

[77]   Petersen, E.J., and Nelson, B.C. Mechanisms and measurements of nanomaterial-induced oxidative damage to DNA. *Anal Bioanal Chem*, 2010. 398(2): p. 613-50.

[78]   Yang, J., et al. Dissociation of double-stranded DNA by small metal nanoparticles. *J Inorg Biochem*, 2007. 101(5): p. 824-30.

[79]   Chen, M., and von Mikecz, A. Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO2 nanoparticles. *Exp Cell Res*, 2005. 305(1): p. 51-62.

[80]   Baweja, L., et al. C60-fullerene binds with the ATP binding domain of human DNA topoiosmerase II alpha. *J Biomed Nanotechnol*, 2011. 7(1): p. 177-8.

[81]   Gupta, S.K., et al. Interaction of C60 fullerene with the proteins involved in DNA mismatch repair pathway. *J Biomed Nanotechnol*, 2011. 7(1): p. 179-80.

[82]   Beyersmann, D., and Hartwig, A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Arch Toxicol*, 2008. 82(8): p. 493-512.

[83]   Hahn, A., et al. Cytotoxicity and ion release of alloy nanoparticles. *J Nanopart Res*, 2012. 14(1): p. 1-10.

[84]   Baldwin, E.L., J.A. Byl, and Osheroff, N. Cobalt enhances DNA cleavage mediated by human topoisomerase II alpha in vitro and in cultured cells. *Biochemistry*, 2004. 43(3): p. 728-35.

[85] Colognato, R., et al. Comparative genotoxicity of cobalt nanoparticles and ions on human peripheral leukocytes in vitro. *Mutagenesis*, 2008. 23(5): p. 377-82.

[86] Westbrook, A.M., et al. Intestinal inflammation induces genotoxicity to extraintestinal tissues and cell types in mice. *Int J Cancer*, 2011. 129(8): p. 1815-25.

[87] Downs, T.R., et al. Silica nanoparticles administered at the maximum tolerated dose induce genotoxic effects through an inflammatory reaction while gold nanoparticles do not. *Mutat Res*, 2012. 745(1-2): p. 38-50.

[88] Tsuda, H., et al. Toxicology of engineered nanomaterials - a review of carcinogenic potential. *Asian Pac J Cancer Prev*, 2009. 10(6): p. 975-80.

[89] Park, E.J., et al. Inflammatory responses may be induced by a single intratracheal instillation of iron nanoparticles in mice. *Toxicology*, 2010. 275(1-3): p. 65-71.

[90] Moller, P., et al. Oxidative stress and inflammation generated DNA damage by exposure to air pollution particles. *Mutat Res Rev Mutat Res*, 2014. 762C: p. 133-166.

[91] Shi, H., et al. Titanium dioxide nanoparticles: a review of current toxicological data. *Part Fibre Toxicol*, 2013. 10: p. 15.

[92] Pfuhler, S., et al. Genotoxicity of nanomaterials: refining strategies and tests for hazard identification. *Environ Mol Mutagen*, 2013. 54(4): p. 229-39.

[93] OECD. *Six years of OECD work on the safety of manfactured nanomaterials: Achievements and Future Opportunities.* 2012. http://www.oecd.org/science/nanosafety/Nano %20Brochure%20Sept%202012%20for%20Website%20%20(2).pdf.

[94] OECD. *Genotoxicity of Manufactured Nanomaterials : Report of the OECD expert meeting.* OECD Environment, Health and Safety Publications 2014. Series on the Safety of Manufactured Nanomaterials No. 43-ENV/JM/MONO(2014)34(http://www.oecd.org/ officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)34&doclanguage=en).

[95] FDA. *Nanotechnology.* Science & Research 2014; Available from: http://www.fda.gov/ ScienceResearch/SpecialTopics/Nanotechnology/default.htm.

[96] FDA. *Use of Nanomaterials in Food for Animals (Draft Guidance).* Guidance for Industry #220, 2014. http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM401508.pdf.

[97] Alotaibi, A., et al. Tea phenols in bulk and nanoparticle form modify DNA damage in human lymphocytes from colon cancer patients and healthy individuals treated in vitro with platinum-based chemotherapeutic drugs. *Nanomedicine (Lond)*, 2013. 8(3): p. 389-401.

[98] Kumar, A., and Dhawan, A. Genotoxic and carcinogenic potential of engineered nanoparticles: an update. *Arch Toxicol*, 2013. 87(11): p. 1883-900.

[99] Lockman, P.R., et al. Nanoparticle surface charges alter blood-brain barrier integrity and permeability. *J Drug Target*, 2004. 12(9-10): p. 635-41.

[100] Shvedova, A.A., et al. Long-term effects of carbon containing engineered nanomaterials and asbestos in the lung: one year postexposure comparisons. *Am J Physiol Lung Cell Mol Physiol*, 2014. 306(2): p. L170-82.

[101] Boland, S., Hussain, S., and Baeza-Squiban, A., Carbon black and titanium dioxide nanoparticles induce distinct molecular mechanisms of toxicity. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 2014. 6(6): p. 641-52.

[102] Demir, E., Creus, A., and Marcos, R. Genotoxicity and DNA repair processes of zinc oxide nanoparticles. *J Toxicol Environ Health A*, 2014. 77(21): p. 1292-303.

[103] Gong, C., et al. SiO(2) nanoparticles induce global genomic hypomethylation in HaCaT cells. *Biochem Biophys Res Commun*, 2010. 397(3): p. 397-400.

[104] Guo, D., et al. Zinc oxide nanoparticles induce rat retinal ganglion cell damage through bcl-2, caspase-9 and caspase-12 pathways. J Nanosci Nanotechnol, 2013. 13(6): p. 3769-77.

[105] Roy, R., et al. Zinc oxide nanoparticles induce apoptosis by enhancement of autophagy via PI3K/Akt/mTOR inhibition. Toxicol Lett, 2014. 227(1): p. 29-40.