KEMI
Swedish Chemicals Agency

Nanomaterials and genotoxicity

– a literature review

REPORT 13/16

kemikalieinspektionen.se

KEMI
Swedish Chemicals Agency

The Swedish Chemicals Agency is supervisory authority under the Government. We work in Sweden, the EU and internationally to develop legislation and other incentives to promote good health and improved environment. We monitor compliance of applicable rules on chemical products, pesticides and substances in articles and carry out inspections. We also provide guidance regarding enforcement and inspections to municipalities and county administrative boards. We review and authorise pesticides before they can be used. Our environmental quality objective is A Non-toxic Environment.

ISSN 0284-1185. Article number: 361 218.

Preface

The Swedish Chemicals Agency has been assigned by the Swedish Government to produce a national action plan for a toxic-free everyday environment: Action plan for a toxic-free everyday environment 2011 – 2014 – protect the children better. The work on the action plan has been extended until 2020.

Efforts are now going on in several areas, both in Sweden, within the EU and internationally and often in cooperation with other authorities. Reducing chemical risks in the everyday environment is one step towards attaining the Swedish Parliament´s environment quality objective A Non-Toxic Environment, which is the objective that we are responsible for.

Within the framework of the action plan, the Swedish Chemicals Agency compiles knowledge in our report and PM series elaborated by experienced colleagues, researchers or consultants. In this way, we present new and essential knowledge in publications which can be downloaded from the website www.kemikalieinspektionen.se

This report/PM has been produced within the framework of the government assignment to carry out the strategy on a non-toxic everyday environment and reaching the environmental quality objective A Non-Toxic Environment 2015–2017.

The report was written by Sebastiano di Bucchianico and Hanna Karlsson at the Institute of Environmental Medicine (IMM), Karolinska Institute.

Project leaders and contacts at the Swedish Chemicals Agency were Lena Hellmér and Lisa Anfält.

Contents

Summary

Nanomaterials (NMs) and nanoparticles (NPs) are currently used in many applications in society and the manufacturing and use is expected to steadily increase in the future. Therefore, risk assessment of these materials is urgently needed. Genotoxic and mutagenic effects need to be carefully evaluated due to the relation to diseases such as cancer as well as the risk of inherited genetic damage.

In this literature review, genotoxicity caused by nanoparticles and underlying mechanisms is discussed, as well as the applicability of the different methods used for genotoxicity testing of nanoparticles. In particular the use of comet assay, micronucleus (MN) assay, chromosome aberration test, bacterial and mammalian mutagenicity tests and cell transformation assays is described. Possible interference of the NPs and the assays is also discussed. For the comet assay, for example, a risk for overestimation of the DNA damage has been suggested when high concentrations of reactive NPs are tested *in vitro* due to additional damage formed during the assay performance. For micronucleus assay treatment with Cytochalasin-B (in order to score MN in once-divided cells) can affect NP-uptake and therefore, delayed co-treatment is recommended. One important question for all NP studies is dosimetry consideration and the fact that the real cell dose is seldom measured. Indeed, bacterial cells have limited ability to engulf NPs and thus, mammalian cells are recommended for mutagenicity testing.

The *in vivo* genotoxicity studies found in the literature for some of the most common NMs were compiled in this review, *i.e*; silicon dioxid (SiO_2), titanium dioxide (TiO_2), gold (Au), silver (Ag) and carbon nanotubes (CNTs). For all materials, both positive and negative studies were reported. It was striking, however, that following administration via the lung, no effects on blood or bone marrow cells were in general observed. In contrast, local effects in lung cells were observed convincingly for CNTs, but not for the other NPs. For TiO_2, several studies showed genotoxicity following oral exposure. Both Au and Ag NPs were also genotoxic following injections, and convincingly positive genotoxicity findings in a range of *in vitro* studies were reported. From the *in vivo* studies it is apparent that the administration route is important when studying the genotoxicity of NMs and a focus on target tissue (e.g. lung following inhalation), is critical.

Sammanfattning

Nanomaterial och nanopartiklar har många användningsområden och tillverkningen väntas öka. Det finns därför ett stort behov av att riskbedöma sådana material och partiklar. Särskilt genotoxiska och mutagena effekter är viktiga att studera på grund av deras samband med sjukdomar såsom cancer och risken för ärftlig genetisk skada.

I denna litteraturstudie diskuteras genotoxicitet orsakat av nanopartiklar och bakomliggande mekanismer samt om vanliga metoder för att studera genotoxicitet kan användas även för att testa nanopartiklar. Framförallt beskrivs kometmetoden, analys av mikrokärnor och kromosomavvikelser, metoder där bakterier och celler från däggdjur används för att testa mutagenicitet, samt celltransformation. Vi diskuterar också om eventuella interaktioner mellan metoderna och nanopartiklarna förekommer och hur det kan påverka resultatet. För kometmetoden har exempelvis en risk för att överskatta DNA-skada visats, när höga koncentrationer av reaktiva nanopartiklar testats *in vitro*. Ett annat exempel är att den vanliga behandlingen med cytokalasin-B vid analys av mikrokärnor (ämnet används för att identifiera celler som delat sig) kan påverka upptaget av nanopartiklar. I det fallet är det därför viktigt att tillsätta cytokalasin-B först efter att cellerna har exponerats för nanopartiklar en tid. En utmaning att ta hänsyn till i de flesta testmetoder är vilken den faktiska dosen i cellerna egentligen blir efter exponering. Exempelvis har bakterieceller en begränsad förmåga att ta upp nanopartiklar och därför är däggdjursceller ett bättre alternativ för att testa mutagenicitet.

I rapporten sammanställer vi även forskning om genotoxicitet *in vivo* för några av de vanligaste nanomaterialen, däribland kiseldioxid (SiO_2), titandioxid (TiO_2), guld (Au), silver (Ag) och kolnanorör (CNTs). För alla material har både positiva och negativa effekter rapporterats. Det är dock slående att efter exponering via lungorna så har generellt sett inga effekter på blod eller benmärgsceller kunnat påvisas. Däremot har flertalet studier visat lokala effekter i lungceller efter exponering för CNTs, men generellt inte för andra nanopartiklar. För nanopartiklar av TiO_2 har flera studier visat genotoxiska effekter efter oral exponering. Även för nanopartiklar av silver och guld har genotoxiska effekter visats, i detta fall efter injektion i blodet på försöksdjur. Genotoxicitet har även visats när guld och silver studerats *in vitro*. Sammanfattningsvis visar studier *in vivo* att exponeringsvägen kan ha en avgörande betydelse för resultatet.

1 Introduction

1.1 What is special with nanoparticles?

Nanomaterials (NMs) are currently utilized in many applications in society and the manufacturing and use is expected to steadily increase in the future. NMs can be described as materials "with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale", where the "nanoscale" often is considered to be 1–100 nm. However, it should be noted that there are variations of this definition and an on-going discussion regarding how these materials best should be defined. Nanoparticles (NPs), as a sub-category of NMs, can be defined as particles with all three external dimensions in the range 1–100 nm although, again, variations on this definition exist. The reason for the increased production and use of NMs is their special physicochemical properties, and there is a hope that these materials will offer improved performances and new functionalities leading, e.g., to smart drugs (Karimi et al, 2016) and aiding in achieving sustainable development, e.g., by reducing the consumption of energy and materials and reducing environmental contamination (Stark et al, 2015). At the same time, there is currently a concern regarding the potential hazardous effects of NMs on human health and the environment.

Indeed, it has often been stated that the same properties that make NPs exciting for technological research and development, also make them problematic from a toxicological perspective. It seems plausible that a reduction in particle size that can improve and accelerate reactions in the case of catalysis or other chemical processes, also leads to increased reactivity with cells. Small particles have clearly a high surface area or "number of particles" given the same mass. For example, considering particles with three different diameters of 1 μm, 100 nm, and 10 nm of a particular material of unchanged mass, the specific surface of these particles increases each decimal step by a factor of 10, and the number of particles even increases by a factor of 1000 (Krug and Wick, 2011). As a consequence, there are considerably more atoms available on the particle surfaces for smaller particles, and they can interact with the environment much more efficiently. In addition, the small size may also imply higher chemical reactivity, not only by the large number of reaction partners on the surface, but also due to surface effects including crystal lattice defects as a consequence of the enormous curvature of the particles (Nel et al., 2009). One other important aspect of (nano)particles is the "transport principle" (Krug and Wick, 2011), or the "Trojan horse effect". The transport of metal ions across the cell membrane is well-regulated, but when the cells meet the metal as a nanoparticle this regulation is circumvented via various endocytotic mechanisms. Many metal and metal oxide nanoparticles can then undergo dissolution within acidic compartments in the cell, which could drive toxicity. Thus, the particle structure acts as a "Trojan horse" and allows toxic ions to "sneak" into cells (Stark, 2011; Cronholm et al., 2013).

1.2 Translocation from lung and GI-tract?

Except from these properties, one reason for concern is that nanoparticles are deposited deeper into the lungs when compared to larger particles (Geiser and Kreyling, 2010). Furthermore, whereas particles deposited at the ciliated airways often are cleared rather rapidly, the retention in the alveolar region is often longer and clearance depends to a high degree on alveolar macrophages. Nanoparticles in the alveolar region can be cleared through two major mechanisms: (1) macrophage-mediated transport to the airways and mucociliary escalation to larynx and pharynx, allowing nanoparticles to be swallowed, transported through

the GI tract, and subsequently excreted in the feces and (2) translocation through the air-blood barrier and via lymphatic vessels of the lungs to eventually reach the bloodstream, and hence to secondary target organs (Geiser and Kreyling, 2010). To what extent nanoparticles can be translocated from the site of deposition (often lung) to the systemic circulation is an important question when performing risk assessments. Animal studies have shown that there is evidence for translocation across the air-blood barrier of e.g. gold, silver, and TiO_2 nanoparticles in the size range of 5-100 nm, but the translocated particle fractions seldom exceeded 5% of the delivered lung dose (Geiser and Kreyling, 2010). When considering reliable studies in humans, there is so far no evidence for a translocated nanoparticle mass fraction of more than 1% of the dose delivered to the lungs. Thus, presently is not clear if this rather low amount of translocation has implications for adverse health effects in humans. It is possible that accumulation in secondary organs may occur after chronic exposure over extended time periods. Indeed, nanoparticles can also be of systemic concern in the absence of translocation due to in systemic inflammation, release of soluble species as well as modulation of the autonomous nervous system leading to subsequent health effects (Kreyling et al. 2013). Following oral exposure, it appears as if NP reaching the GI tract are mostly excreted with the feces, but some NM absorption of low levels which became systemically available has been observed (Landsiedel et al, 2009). As reviewed by Braakhuis et al (2015) several studies have investigated translocation of TiO_2 NPs in in vivo models as well as in vitro and the translocation is often under detection. For example, six hours after a single oral administration of 130-nm TiO_2, some titanium could be detected in gut tissues, but the levels were too low to allow for quantification (Brun et al. 2014). In another study, rats were exposed to 5 mg/kg of different types of TiO_2 NPs (mean particle size 40 nm, 40–50 nm, 120 nm and up to 5 µm), and up to 96 h post-administration, no translocation of titanium was detected to blood, several organs and urine. Also, no translocation was observed *in vitro* (MacNicoll et al. 2015). In addition to TiO_2 NPs, also SiO_2 is commonly used as food additive. In one study limited uptake was observed after 28 and 90 days of exposure to food-grade synthetic amorphous silica (van der Zande et al. 2014).

Taken together there are many reasons for being concerned over an increase in nanoparticle exposure, especially via inhalation. It should, however, be noted that the question of whether or not nanoparticles exhibit novel mechanisms of toxic action is currently a subject of considerable debate (Donaldson and Poland, 2013).

In the next section, there will be a focus on genotoxicity and underlying mechanism

2 Nanoparticle-induced genotoxicity and underlying mechanisms

The detailed mechanisms of nanoparticle-induced genotoxicity are not completely understood and it is furthermore not clear if there are any nano-specific effects on DNA (Donalson and Poland). The “nano-specific effect” refers to mechanism of toxic action that is specific to particles with initial dimensions within the size range 1–100 nm as opposed to also being associated with particles of different sizes but with the same chemical composition. In general, particle induced genotoxicity can be classified as either “primary genotoxicity” or “secondary genotoxicity”, where primary genotoxicity refers to genotoxicity from the nanoparticles themselves whereas “secondary genotoxicity” refers to the induction of genotoxicity via reactive oxygen species (ROS) generated during particle-elicited inflammation (Schins and Knaapen 2007).

2.1 Primary genotoxicity – direct and indirect mechanisms

If NPs enter the cell nucleus, either by penetration via nuclear pores or during mitosis, they might directly interact with DNA. Direct DNA interaction could represent a more nano-specific mechanism due to the fact that small NPs may reach the nucleus via transportation through the nuclear pore complexes (NPC) (Nabiev et al., 2007). NPCs are the only channels through which small polar molecules, macromolecules and NPs are able to travel through the nuclear envelope and it consist of a tube with a diameter of approximately 30 nm. It has therefore been reported that particles larger than 30 nm can only be transported through the pores when tagged e.g. with nuclear localization sequence (NLS). However, also larger nanoparticles of e.g. silver (60 nm) (Kim et al., 2011) SiO_2 (40–70 nm) (Chen et al., 2005) and CuO (50–100 nm) (Wang et al, 2012) have been observed in the nucleus suggesting that larger NPs may get access to the DNA in dividing cells when the nuclear membrane disassembles. If NPs interact or bind with DNA molecules this could influence DNA replication and transcription of DNA into RNA. To study this, Li and co-workers (Li et al., 2013) proposed that DNA-binding assays can be useful and showed that NPs (size range 3–46 nm) with a high affinity for DNA strongly inhibited DNA replication (tested acellularly), whereas NPs with low affinity had no or minimal effect. Clearly, such experimental acellular studies do not consider important factors such as the ability of the NPs to enter the nucleus, and the fact that DNA is highly packed in mammalian cells. The likelihood for nuclear localization and DNA interaction depends on the NP size as well as its charge. For example Nabiev et al. demonstrated that green (2.1 nm) quantum dots (QDs) but not red ones (3.4 nm) entered the nucleus of THP-1 cells via nuclear pore complexes (Nabiev et al., 2007). One novel way of studying direct DNA interaction resulting in stalled replication forks is to use reporter cells sensitive to such effects. This approach was used in a study testing different metal oxides as well as Ag NPs of different sized (Karlsson et al, 2014). This study showed, however, no evidence for direct DNA interaction leading to stalled replication forks by any of the tested NPs. Instead, reporter cells showing oxidative stress were activated mainly by CuO and NiO NPs (Karlsson et al, 2014). In one of the few studies claiming a size dependent interaction with DNA, gold NPs with a distinct particle size of 1.4 nm were shown to interact in a unique manner with the major grooves of DNA, which could account for the toxicity of these small NPs (Tsoli et al., 2005).

Except from direct DNA interaction, there are several other mechanisms leading to genotoxicity. Probably the most reported effect related to genotoxicity is oxidative stress. For mechanistic purposes, one should discriminate between the oxidant-generating properties of particles themselves (i.e., acellular), and their ability to stimulate cellular oxidant generation (Knaapen et al., 2004). ROS can result from reactions at the surface of the nanoparticles or via release of redox-active transition ions such as Fe^{2+}, Ag^{+}, Cu^{+}, Mn^{2+}, and Ni^{2+} leading to the production of ROS via the Fenton-type reaction. An example of stimulation of cells to release ROS is an interaction with the mitochondria that may affect the electron transport chain or ROS formation via induction of P450 enzymes. Nanoparticles may also affect proteins involved in DNA repair or in antioxidant response, also leading to genotoxicity via indirect mechanisms (Magdolenova et al, 2013). For example Wojewódzka et al. (2011) found that treatment with Ag NPs delays repair of X-ray-induced DNA damage in HepG2 cells and Jugan et al. (2011) reported that TiO_2 NP impaired cellular DNA repair through inactivation of BER and NER pathways.

Another possibility is that NPs interact with the mitotic spindle apparatus, centrioles or their associated proteins and thereby cause aneugenic effecs, i.e. loss or gain of chromosomes in daughter cells. As summarized by Sargent et al (2010), the long thin tubular-shaped carbon

nanotubes have a striking similarity to cellular microtubules, suggesting a potential to interact with the mitotic spindle as well as the motor proteins that separate the chromosomes during cell division. Such a disruption of centrosomes and mitotic spindles would result in monopolar, tripolar, and quadrapolar divisions of chromosomes resulting in aneuploidy (Sargent et al, 2010). Aneugenic effects have also e.g. been reported for CuO and gold NPs (Di Bucchianico et al., 2013 and 2014).

Except from these mechanisms, other more indirect mechanisms have been suggested. Bhabra et al (2009) showed that cobalt–chromium NPs could damage human fibroblast cells across an intact cellular barrier without having to cross the barrier. It was shown that the damage was mediated via transmission of purine nucleotides (such as ATP) and intercellular signalling within the barrier through connexin gap junctions or hemichannels and pannexin channels.

2.2 Secondary (inflammation-induced) genotoxicity

As a contrast to primary genotoxicity, secondary genotoxicity can be defined as genetic damage resulting from reactive oxygen/nitrogen species (ROS/RNS) (and possibly other mediators) that are generated during particle-elicited inflammation from activated phagocytes (macrophages, neutrophils). As discussed e.g. by Schins and Knaapen (2007) as well as Borm et al (2011) one important factor for risk assessment is that secondary genotoxicity is considered to involve a threshold. This threshold level is determined by the exposure concentration that will trigger inflammation and overwhelm antioxidant and DNA damage repair capacities in the lung. Since the ability of particles to elicit inflammation vary depending on many properties (particle solubility, surface reactivity etc.), the threshold will also vary between different NPs. For crystalline silica, the secondary inflammation-driven genotoxicity mechanism is generally accepted and recognized as an important mechanism for the carcinogenic action (Borm et al, 2011).

3 Genotoxicity assessment of nanomaterials

A regulatory test battery for genotoxicity consist often of 1) an *in vitro* test for mutations in bacteria, 2) an *in vitro* test for cytogenetic effects, micronuclei, or mutations in mammalian cells. In some cases (depending on the results of the 1 and 2) also *in vivo* test such as micronuclei in erythrocytes or chromosomal aberrations in bone marrow cells will be needed. Test methods that are often included *in vitro* is OECD 471: "Bacterial Reverse Mutation Test", OECD 473: "In vitro Mammalian Chromosome Aberration Test", OECD 487: "In Vitro Mammalian Cell Micronucleus Test" and the OECD 476: "In vitro Mammalian Cell Gene mutation Test ". If considering the scientific literature in general, the most commonly used assays for assessing genotoxicity of NPs is the comet assay and analysis of micronuclei *in vitro* (Magdolenova et al, 2014; Golbamaki et al, 2015). In the following section, the applicability of the different methods used for genotoxicity testing of NPs is discussed, in particular the comet assay, micronucleus (MN) assay, chromosome aberrations, bacterial and mammalian mutagenicity tests and cell transformation assays.

3.1 Comet assay

This assay is the most used assay used for assessing genotoxicity of nanoparticles. In 2014, Magdolenova et al (2014) found 58 *in vitro* and 9 *in vivo* studies and many more studies have been published since then. As reviewed previously, most of the studies show positive outcomes (Karlsson 2010, Magdolenova et al., 2014; Golbamaki et al, 2015).

3.1.1 Introduction to the method

The comet assay or "single-cell gel electrophoresis" was first described in 1984 by two Swedish researchers, Östling and Johanson (1984). A few years later Singh et al. (1988) introduced an alkaline version (pH >13) of the method that today is the most widely used. This version of the assay enables the detection of single-strand breaks (SSB), directly produced or associated with incomplete excision repair, as well as alkali-labile sites. The latter include abasic sites that arise from the loss of a damaged base from the sugar in the DNA backbone. Such sites can arise spontaneously due to damage in the bases or in the sugars, or as intermediates during base excision repair.

The assay starts with embedding the cells (following exposure) in low-melting-point agarose gel on a microscope slide. After the gels have solidified, the slides are placed in a lysis solution containing Triton X-100 (in order to break down membranes) and a high concentration of salt (2.5 M NaCl), which will remove histones and other soluble proteins. After this step, the supercoiled DNA is attached to a nuclear matrix creating a structure that has been called a "nucleoid." The slides are then incubated in alkaline electrophoresis buffer leading to DNA unwinding and electrophoresis is then performed under the same alkaline conditions, typically for approximately 20–30 min at 0.7–1.15 V/cm. The electric field causes the negatively charged damaged DNA to migrate toward the anode. This results in an image that looks like a comet with a head and a tail. The more strand breaks that are present in DNA, the more DNA will be present in the tail. After neutralization, the slides are stained after which the comets are analyzed by fluorescence microscopy using an image analysis system. The image analysis calculates different parameters for each comet, the most often used being tail length, percentage DNA in the tail (% tail DNA), and the so-called tail moment (calculated as tail length × the total tail intensity). In general, % tail DNA is regarded as easier to interpret and more useful, and its use is therefore recommended. The comet assay can also be modified in order to allow the specific detection of oxidatively damaged DNA. The principle is, following the lysis step, a lesion-specific endonuclease is added that removes the damaged base creating an abasic site that, via the lyase activity of the enzyme, or by the subsequent alkaline treatment, is transformed to a strand break. The most commonly used enzymes are formamidopyrimidine DNA glycosylase (FPG), which recognizes oxidatively damaged purines, mainly 8-oxoguanine, and formamidopyrimidines (ring-opened purines), or endonuclease III (EndoIII) that nicks the DNA at sites of oxidized pyrimidines (Collins, 2011). The difference in tail intensity between cells treated with enzymes and those not treated (net FPG or Endo III sites) gives a measure of the amount of oxidatively damaged DNA.

3.1.2 Can the method be applied to nanomaterials?

Some concerns for interactions of nanoparticles within the comet assay have arisen from the observation that NPs can be observed in the "comet head" following exposure to high doses *in vitro* (Stone et al., 2009; Karlsson, 2010). This implies that NPs may be present during performance of the assay, which leads to the question if such NPs may induce additional breaks in "naked DNA" during the assay performance, i.e. breaks that were not induced in the cell (potentially resulting in false high levels of damage). Indeed, in a recent study (Karlsson et al., 2015), it was found that a substantial amount of DNA damage was observed in cells only exposed to CuO during the assay performance (added in the last wash-step). This shows that DNA-damaging particles present during the assay can cause additional DNA damage. NPs that may be present during the assay are most likely mainly the intracellular ones (i.e. those in the cytosol). An important question that follows is whether CuO-induced damage

observed in the comet assay is “artificial” and mainly induced during the assay. Most likely this is not the case according to a number of studies which show, for example, genotoxicity using other assays (Bucchianico et al., 2013; Karlsson et al, 2014) as well as up-regulation in expression of DNA damage-inducible genes 45 β and γ (GADD45B and GADD45G) (Hanagata et al., 2011). In a recent study, Ferraro et al (2016) also reported evidence of overestimation of NP genotoxicity by the comet assay in the case of CeO_2, TiO_2, SiO_2, and polystyrene NPs. The overestimation was particularly obvious for TiO_2 nanoparticles, although very high doses of nanoparticles were used (50 and 200 mg/mL).

Taken together, it seems like there is a risk of overestimating the DNA damage when high doses of reactive nanoparticles are being used in *vitro*. However, as will be discussed more in section 3.3, there are often rather good consistency between comet assay and micronucleus assay. Such a correlation seems unlikely if pronounced false positive results are gained from the comet assay. Probably, there is rather a quantitative difference (overestimation) rather than qualitative (false positives). Possibly one exception is photocatalytically active nanoparticles such as TiO_2 that may cause false positives due to catalytic activation by the lab light (Karlsson et al, 2015). Overall, avoiding to test at very high doses (e.g. >50 mg/mL) also decreases the risk of overestimating the DNA damage.

3.2 Micronucleus assay

This assay is the second most used assay used for assessing genotoxicity of NPs with 31 *in vitro* and 14 *in vivo* studies published 2014 (Magdolenova et al., 2014) and several more published since then.

3.2.1 Introduction to the method

Micronuclei (MN) mainly originate from acentric chromosomes and/or chromatid fragments or whole chromosomes that fail to be incorporated in the daughter nuclei at the end of telophase during mitosis due to spindle defects during the segregation process in anaphase (Fenech et al., 2011). MN containing chromosomes or chromosome/chromatid fragments are enclosed by a nuclear membrane showing similar morphology to nuclei after conventional nuclear staining, except for their smaller size, which is between 1/18 and 1/3 of that of the main nucleus. The MN assay detects both chromosome breakage (clastogenicity for example induced by ROS) as well as aneuploidogenic effects that can be due to physical disturbance of spindle/mitotic apparatus (Pfuhler et al., 2013). The OECD test guideline 487 defines the in vitro mammalian Cell Micronucleus (MN) test while the in vivo mammalian Erythrocyte Micronucleus test is defined in the OECD test guideline 474 (OECD 487, OECD 474).

3.2.2 Can the method be applied to nanomaterials?

In the cytokinesis-block micronucleus (CBMN) assay, Cytochalasin-B (Cyt-B) is used to score MN specifically in once-divided binucleated cells. This use may, however, interfere with the uptake of particles. For example Papageorgiou et al (2007) showed using CoCr alloy NPs that the level of chromosomal damage was higher following delayed exposure to Cyt-B (*i.e.* first exposure in the absence of Cyt-B) than in cells co-exposed to and Cytochalasin B. Similar findings were observed by Lindberg et al (2013) in a study on BEAS-2B cells exposed to SWCNTs and MWCNTs (Lindberg et al, 2013). Thus, in order to avoid decreased cellular uptake as a consequence of actin inhibition by Cyt-B, post-treatment or delayed co-treatment is suggested for genotoxicity testing of NPs (Gonzalez et al, 2011).

3.3 Comet vs micronucleus in vitro

Indeed, many published studies report DNA strand breaks caused by NPs, and this reflects likely the high sensitivity of the assay together with high reactivity of these NPs (and possibly also some publication bias in favor of positive results) (Karlsson, 2010). In view of this, the comet assay is sometimes criticized for giving positive response "too frequently". One way of deciding whether the comet assay is a relevant assay to test for NP-induced genotoxicity is to investigate whether genotoxicity is also observed with other assays. Differences in sensitivity and the fact that different assays measure different types of damage mean that perfect agreement is unlikely. In a recent study (Karlsson et al, 2015), studies combining comet assay and MN-assay were examined to check for the level of agreement between them. It was found that the comet and micronucleus assays were used simultaneously to describe the genotoxicity of 66 nanomaterials (some materials such as TiO_2 P25 were tested in several studies and hence there were not 66 completely different materials) in 38 papers. It was found that 81% of the materials were positive in the comet, while 57% showed positive micronucleus induction (57%), with consistent results for 69% of the materials. A comparison based on chemical composition revealed a higher rate of inconsistency between comet and MN results for TiO_2 NPs. In fact, 19 of 22 analyzed TiO_2 NPs induced DNA strand breakage (86%), while only 7 showed positive MN results (32%) and 9 showed consistent results (41%). When studies on TiO_2 NPs were excluded, the consistency between comet and MN-assay results was 81%. Thus, an overall good consistency was observed for comet and NM-assay in the *in vitro* studies published to date. The consistency was, however, not very solid for TiO_2 NPs showing higher number of positive outcomes for comet assay.

3.4 Chromosome aberrations

3.4.1 Introduction to the method

The OECD guidelines TG 473 and TG 475 define *in vitro* mammalian chromosome aberration test and *in vivo* mammalian bone marrow chromosomal aberration test, respectively (OECD 743, OECD 475). The chromosome aberration (CA) test identifies agents that cause chromatid-type or chromosome-type breakage and exchanges, dicentric chromosome formation, endoreduplications and other abnormal chromosomes, notably translocations which are implicated in the aetiology of various human genetic diseases and cancers. For *in vitro* and *in vivo* testing, cell cultures or animals (generally rodents) are exposed to the test substance and treated with a metaphase-arresting substance (e.g. colcemid) to accumulate metaphase cells. Chromosome preparation are then made from cultured cells or bone marrow cells and methaphase cells are analyzed microscopically (Galloway et al. 1994; Tice et al, 1994; Mosesso et al, 2013).

3.4.2 Can the method be applied to nanomaterials?

There is no evidence for *in vitro* CA test interactions with NPs whereas if there is evidence that the NPs will not reach the target tissue *in vivo*, it may be not appropriate to use CA test in bone marrow cells.

3.5 Bacterial mutagenicity test

3.5.1 Introduction to the method

The Ames test (bacterial reverse mutation) (OECD, 1997) is based on induction of back-mutations in a defective histidine gene; reversal of this mutation will enable the bacterium to synthesise histidine and form a visible colony when plated in minimal histidine medium. Typically, as recommended in OECD Test Guideline no. 471, one or more strains of *Salmonella (S. typhimurium)* and/or *Escherichia coli* are used, e.g. the *S. typhimurium* strains TA97a, TA98, TA100, TA102, TA1535 and TA1537 or the E. coli strain WP2uvrA. It can also be used with or without metabolic activation, *i.e.* typically with or without "S9-mix". In short, a typical Ames test involves exposing the bacterial strain to a test agent and then placing the exposed bacteria in petri dishes that contain agar with no histidine. After incubating the dishes, the bacteria that have grown are counted. This number, which reflects the bacteria that undergo a reverse mutation from is compared to the number of bacteria that undergo reverse mutations when they are not exposed to the agent. If the agent causes too many reverse mutations above those measured as spontaneous, it is considered to be mutagenic.

3.5.2 Can the method be applied to nanomaterials?

There are concerns regarding the suitability of the Ames test for NP testing, the main reason being that bacteria cells do not have any endocytotic mechanisms for uptake of particles and it is therefore suspected that nanoparticles may not able to penetrate the rigid outer double membrane of Gram negative bacteria. There are, however, several examples of studies showing uptake. Kumar et al., (2011) used flow cytometry and TEM imaging and showed uptake as well as a weak mutagenic potential of of ZnO and TiO_2 nanoparticles. The authors suggested that the protein coat obtained when using S9 facilitated entry of NPs into the cells. In another study, Clift et al (2013) used the TA98 *S. typhimurium strain*, which is the most commonly used strain for the Ames test, and tested the interaction with various nanoparticles. It was found that the nanomaterials including CeO_2 NPs, SWCNTs and MWCNTs, were able to enter the bacteria cells. Still, the results on mutagenicity were negative (Clift et al., 2013). Other studies show lack of uptake; Butler et al (2014) found that TiO_2 nanoparticles were only attached to the surface of the bacteria cells and were never internalized (Butler et al., 2014). Similarly, when AgNPs of different sizes (10, 20, 50 and 100nm) were tested, no uptake was observed as well as no mutagenicity (Butler et al., 2015). As summarized in previous reviews (Landsiedel et al.,2009; Magdolenova et al, 2014), most studies on mutagenicity show negative results. There are, however, also examples of positive studies outcomes. As already discussed Kumar et al (2011) showed mutagenic potential of of ZnO and TiO_2 NPs. Whereas ZnO mainly were mutagenic in presence of S9 fraction, the mutagenic potential of TiO_2 NPs was independent of metabolic activation system. Pan et al (2009) showed negative mutagenicity outcome for Al_2O_3, Co_3O_4, TiO_2, and ZnO when tested up to 1000 μg/plate. A slight increase in mutagenicity was, however, noted for one of the strains (*Escherichia coli WP2 trp uvrA*) when using high S9 activation (Pan et al, 2009). Furthermore, in another study (Gooma et al, 2013) a slight increase in mutagenicity in one out of four strains was observed for Fe_3O_4 NPs, but only in the highest dose tested and with S9 activation. In another study on Fe_3O_4 NPs, it was concluded that the mutagenicity dependent both on the size of the NPs as well as the coating. PEG-coated Fe_3O_4 NPs with a size of 10 nm were mutagenic in all strains tested (TA97 TA98 TA100 TA102 TA1535) independent on

S9, whereas 30 nm-sized only were positive in the presence of S9 (Liu et al, 2014). In contrast, NPs with a positive charge (SEI-coated), were not mutagenic.

Taken together, although there are examples of studies showing NP-uptake in bacterial cells used for mutagenicity tests, several studies show also lack of. In several cases a positive outcome was observed only in the presence of S9. Still, the lack of uptake indicates that the use of mammalian cells is a better choice for testing mutagenicity of nanoparticles.

3.6 Mammalian mutagenicity tests

3.6.1 Introduction to the method

OECD guidelines are available for *in vitro* mutation assays that are able to detect forward mutations in reporter genes. However, except the mouse spot test (OECD 484) never used for nanoparticles induced mutation, test guidelines for detecting mutations *in vivo* is currently not available.

In Vitro Mammalian Cell Gene Mutation tests using the hypoxanthine-guanine phosphoribosyl transferase (*Hprt* in rodent cells and *HPRT* in human cells) or the xanthine-guanine phosphoribosyl transferase transgene (*gpt*) (*XPRT*) genes detect different spectra of mutagenic events. While the *HPRT* test detect mutational events like base pair substitutions, frameshifts, small deletions and insertions, the *XPRT* assay may allow also the detection of mutations resulting from large deletions and possibly mitotic recombination due to the autosomal location of the gpt transgene (OECD 476).

Cells are exposed to the test NPs and then sub-cultured for a sufficient period of time to determine cytotoxicity and allow phenotypic expression prior to mutant selection using the purine analogue 6-thioguanine. Mutant frequency is calculated based on the number of mutant colonies corrected by the cytotoxicity at the time of mutant selection (Johnson, 2012).

3.6.2 Can the method be applied to nanomaterials?

So far there is no evidence for interactions of mammalian cell mutation assay in NP testing. The only concern could be related to the persistence of NPs during the clonogenicity assay that could result to an overestimation of cytotoxicity thus impacting the mutation frequency evaluation. However, there is no evidence in this regard.

3.7 Cell transformation

3.7.1 Introduction to the method

To date the standard regulatory approach to assess in vivo carcinogenicity is based on OECD guideline TG 451 (OECD 451). The carcinogenic properties of a test substance are identified by the increasing incidence of neoplastic histopathological findings following 2-years oral, dermal or inhalative administration in rodent species.

Several *in vitro* methods have been developed to evaluate the carcinogenic potential of a test substance. Among these, the cell transformation assays (CTA, Tanaka et al, 2012) and Soft Agar Colony Forming assay (Shoemaker et al, 1985) are the most used in evaluating the carcinogenic potential of NPs.

The CTA uses established cell lines (Balb/3T3 mouse fibroblast (clone A31-1-1), Syrian Hamster Embryo cells or Bhas 42 cell line derived as a clone formed by the stable

transfection of the v-Ha-ras oncogene into the Balb/3T3) to evaluate the ability of test compound to induce morphological neoplastic transformation of treated cells as evaluated by their ability to form foci type III colonies (morphologically transformed colonies). Recently, the development of an OECD test guideline was recommended (Corvi et al, 2012), and a guidance for conducting the in vitro Bhas 42 CTA was recently presented (ENV/JM/MONO(2016)1). The Soft Agar Colony Forming assay measures cells anchorage-independent growth in vitro by manual counting of colonies in semisolid culture media. The assay has been used in studies testing NMs. For example in a study in which the toxicological effects of nude and chemically functionalized (-NH_2, -OH and –COOH groups) MWCNTs were investigated (Ponti et al, 2013). The result showed anability of all types of MWCNTs to induce neoplastic transformation as assessed by CTA in Balb/3T3 mouse fibroblasts (Ponti et al, 2013).

3.7.2 Can the method be applied to nanomaterials?

The OECD guideline 451 does not extend to the evaluation of carcinogenic potential of NPs which can need specific methodological approaches. So far there is no evidence for interactions of *in vitro* cell transformation assays in NP testing.

3.8 High-throughput methods

The assays presented above are usually regarded as quite "low throughput" and there is a need for assays that enable more high-throughput screening. Several such assays and their applicability for testing NMs are described in a recent review (Bryant et al, 2016). Many are based on modifications of current assays such as "CometChip assay" and flow cytometry scoring of micronuclei or g-H2AX foci. Another example is automated "Fluorimetric Detection of Alkaline DNA Unwinding" (FADU) assay. An attractive approach to enable high throughput analyses of a range of NMs is to use reporter cell lines that are constructed to fluoresce upon activation of certain signaling pathways and in order to get insight into different mechanisms of (geno)toxicity, a combination of various reporter cell lines would be required. In order to meet this need, the ToxTracker assay was developed by Hendriks and co-workers (Hendriks et al, 2011). The assay consists of a panel of six mouse embryonic stem (mES) cell lines that each contains a different GFP-tagged reporter for a distinct cellular signaling pathway with focus on DNA damage, oxidative stress, p53-related cellular stress and protein unfolding (Hendriks et al, 2016). The assay was adapted to a 96-well plate format, thus enabling medium/high throughput screening. In a recent study, an extensive validation using the compound library suggested by ECVAM (the European Centre for the Validation of Alternative Methods) was performed and the result showed that the ToxTracker assay had a very good sensitivity and specificity (Hendriks et al, 2016). There are several advantages with using the ToxTracker assay in NMs genotoxicity studies. The mES cells that are used in the ToxTracker assay are untransformed, proficient in all major DNA damage and cellular stress response pathways and have been shown to efficiently engulf NPs (Karlsson et al, 2014). The NPs tested so far have shown various effects, some being highly toxic and efficiently inducing one or several of the reporters and others showing no effects at all. In all, this indicates that the ToxTracker reporter cell assay can be applied as a rapid mechanism-based tool for assessing the potential genotoxic effects of NPs.

4 Genotoxicity testing of different nanomaterials

In the following section, the *in vivo* genotoxicity studies found in the literature for the materials: silicon dioxid (SiO_2), titanium dioxide (TiO_2), gold (Au), silver (Ag) and carbon nanotubes (CNTs), providing examples of some of the most common NMs, will be discussed. In addition, several examples of recent in vitro studies were compiled. As will be obvious for all materials both positive and negative studies were reported.

4.1 SiO_2

Whereas crystalline silica in the form of quartz or cristobalite is carcinogenic to humans (Group 1), amorphous silica is not (Group 3) as evaluated by IARC (1998). As discussed previously, the main mechanism is believed to be sustained inflammation for the inhalation exposure (Borm et al, 2011).

4.1.1 In vivo studies

As summarized in Table I, 3 of 5 *in vivo* studies found in the literature report negative data (Sayes et al, 2010; Guichard et al, 2015, Maser et al, 2015), one reports negative data for most endpoints with one exception (MN in colon in the lowest dose tested) and one study found a small increase in DNA damage following injection of SiO_2 NPs (Downs et al, 2012).

In an inhalation study investigating genotoxicity of amorphous SiO_2 NMs in rats following 1- or 3-day inhalation no increased levels of MN in reticulocytes was observed (Sayes et al, 2010). In rats, the intratracheal instillation of four different synthetic amorphous SiO_2 NPs induced a strong and dose-dependent lung inflammatory response following short-term exposures, but without any significant DNA damage (Guichard et al, 2015). Furthermore, in the same study but using intravenous injections, no DNA damage in various tissues or bone marrow erythrocyte MN increase was observed despite the hepatotoxicity, thrombocytopenia, and even animal death. The authors discussed that accurate assessment of the cytogenetic effects of NPs requires improvements of the current *in vivo* testing methodologies and, in particular, the lack of particulate positive controls limits the relevance of test results. In another study, no DNA damage (comet assay in lung tissue, MN in reticulocytes) was observed following intratracheal instillation of two different sizes of amorphous SiO_2 NPs. In contrast, genotoxic effects in lung cells *in vitro* was observed although at higher concentrations respect to the dose reached in targeted cells *in vivo* (Maser et al, 2015).

In a study using oral exposure to amorphous SiO_2 NPs (Tarantini et al, 2015), no induction of DNA damage (comet and fpg comet) in various organs of rats was observed and neither MN in bone marrow. However, a weak increase of MN in the colon of rats exposed to the lowest dose of pyrogenic amorphous SiO_2 NPs was noticed. The authors discuss the possibility that this outcome might be related to a higher bioavailability of pyrogenic amorphous SiO_2 NPs respect to the precipitated SiO_2 NPs in the lowest dose due to a lower agglomeration/aggregation tendency. The only study showing an increase in DNA damage in blood cells (MN in reticulocytes as well as increased circulating reticulocytes) was a study using intravenous injections of amorphous SiO_2 NPs in rats (Downs et al, 2012). Also, increased DNA damage in liver was found. Since no DNA damage was found in in vitro studies the authors discuss role of inflammatory reactions.

Taken together, the *in vivo* studies on genotoxicity of SiO_2 NPs suggest at this point no effects following inhalation/intratracheal exposure, a small effect in colon following oral exposure as

well as in liver and blood cells following intravenous injections. It should be noted that only after direct injection into blood, a increase in MN in blood cells was observed.

4.1.2 Examples of in vitro studies

Experimental *in vitro* data on SiO_2 NPs genotoxicity are represented in Table II. SiO_2 NPs induced cyto- and genotoxic effects in mouse lung epithelial cells in a size-dependent manner as a consequence of higher internalization and subsequent lysosomal overload respect to micro-sized particles (Decan et al, 2016). However, to verify whether size is the factor that regulates the genotoxic responses is important to consider the role of different dose metrics since at the same mass concentration correspond a different number of NPs and a different surface area. For instance, the role of different dose-metrics was analyzed following A549 lung epithelial cells exposure to differently sized SiO_2 NPs (Gonzalez et al, 2010). In this study, the smallest NPs (16 nm) showed a slight induction of MN whereas considering all SiO_2 NPs together (16, 60, 105 nm), particle number and total surface area appeared to account for chromosomal damage as they both correlated significantly with the amplitude of the effect. Moreover, since NPs surface coating can modify their surface chemistry, uptake and bioactivity, it is expected that serum proteins modulate their toxicity. Gonzalez and collaborators demonstrated that serum had an influence on cell viability, cell cycle changes and MN induction by exposing A549 lung epithelial cells to differently sized SiO_2 NPs either in presence or absence of serum in cell culture medium (Gonzalez et al, 2014). Higher sensitivity in serum-free conditions was shown especially for larger SiO_2 NPs. Furthermore, due to the different carcinogenic risks of crystalline (quartz) versus the amorphous silica (vitreous) as defined by IARC, scientists are still debating if the crystalline structure, per se, can modulate the genotoxic potential of SiO_2 particles. For instance, when A549 cells and murine macrophages RAW264.7 were exposed to pure quartz or amorphous silica, quartz but not amorphous silica induced genotoxicity in murine macrophages whereas A549 cells were relatively resistant to both particles (Guidi et al, 2015). Moreover, amorphous silica particles with different structure and dimension induced cytotoxic and genotoxic effects both in A549 cells and RAW264.7 murine macrophages although to a different extent and showing a different sensitivity of DNA damage versus chromosomal damage in defining induced genotoxic mechanisms (Guidi et al, 2013).

The physical-chemistry characteristics of SiO_2 NPs can thus modulate their cytogenetic effects often exerted through oxidative stress related mechanisms.

4.2 TiO_2

TiO_2 is approved as a white-colored food additive in Europe (E171) (Commission regulation (EU) No 1129/2011). Although E171 is not considered as a nanomaterial according to the EU recommendation (2011/696/EU), exposure and uptake of the nano sized fraction of E171 cannot be excluded. This makes it important to study and understand the tissue distribution and clearance of TiO_2 NPs, not at least in order to properly select target tissues for assessing the genotoxic potential of TiO_2 NPs. TiO_2 NPs with different sizes and crystal structures showed very limited bioavailability after oral exposure even if increased levels of titanium were detected in liver and mesenteric lymph nodes of exposed rats (Geraets et al, 2014). Following intravenous exposures (both single and repeated), titanium rapidly distributed from the systemic circulation to liver, spleen, kidney, lung, heart, brain, thymus, and reproductive organs with liver, spleen and lung identified as the main target tissues (Geraets et al, 2014). In another study, biodistribution of different sized TiO_2 particles in mice after oral administration showed that titanium mainly retained in the liver, spleen, kidneys, and lung

tissues after uptake by gastrointestinal tract, but only a slight increases of titanium content in red cells was noticed, not significantly different from the control group (Wang et al, 2007). Indeed, the question whether TiO_2 is genotoxic *in vivo* is important, not at least since IARC has evaluated TiO_2 as possibly carcinogenic to humans (IARC 2010) since there is sufficient evidence in experimental animals for the carcinogenicity of TiO_2 following inhalation.

4.2.1 In vivo studies

A summary of *in vivo* data is shown in Table III. Of the 11 studies found in the literature, 6 report negative findings and 5 positive. Of four studies using intravenous injection of TiO_2 NPs, three report lack of DNA damage (Sadiq et al, 2012; Luoro et al, 2014 and Suzuki et al, 2016) and one (Dobrzyńska et al, 2014) show positive effects. Although titanium levels can increase in bone marrow cells following repeated intravenous instillations and the levels of titanium were maintained after 48 h of the last treatment, TiO_2 anatase NPs did not induce MN in reticulocytes and were not mutagenic as assessed with Pig-a gene mutation assay (Sadiq et al, 2012). Concordantly, despite the biopersistence of TiO_2 NPs in liver cells 28 days following the last intravenous injection and a moderate inflammatory response, genotoxic effects were not detected in exposed mice neither 28 days after the last exposure (comet assay in spleen and liver cells) nor 48 h later (MM assay in peripheral blood reticulocytes) (Louro et al, 2014). Furthermore, TiO_2 P25 NPs did not significantly increase the frequency of MN in reticulocytes, the frequency of Pig-a mutants in erythrocytes, or the levels of DNA damage in liver tissues as well as showed no mutagenic potential as assessed by *gpt* and *Spi*- mutation assays in liver (Suzuki et al, 2016). However, following a single intravenous injection of TiO_2 anatase/rutile NPs it was shown that different bone marrow cells can have a different susceptibility (Dobrzyńska et al, 2014). Whereas TiO_2 NPs did not induce MN in reticulocytes nor DNA strand breaks in leukocytes, a significant increase of MN frequency in bone marrow polychromatic erythrocytes was shown 24 h post exposure (Dobrzyńska et al, 2014).

Of the four studies using oral/intragastric administration, one was negative (Donner et al, 2016) and three were positive (Chen et al, 2014; Sycheva et al, 2011; Trouiller et al, 2009). The inability of six different TiO_2 NPs (3 pigment-grade and 3 nanoscale) to migrate from the gastrointestinal tract into the blood was reported and no signs of genotoxicity were noticed in peripheral blood reticulocytes in orally-exposed rats (Donner et al, 2016). Oral administration of TiO_2 anatase NPs induced DNA double strand breaks as assessed by γ-H2AX assay (phosphorylated histone H2AX) without increasing the MN frequency in bone marrow cells following 30-day treatment (Chen et al, 2014). Interestingly, a genotoxic investigation of differently sized TiO_2 NPs in six organs of mice after oral gavage treatments showed significant cytogenetic effects likely caused by a secondary genotoxic mechanism associated with inflammation and/or oxidative stress (Sycheva et al, 2011). The bigger particles (160 nm) induced DNA damage and MN in bone-marrow cells whereas the smallest one (33 nm) induced genotoxic effects both in bone-marrow and liver cells. None of the particles induced DNA damage in brain cells and MN or other cytogenetic damage (e.g. nuclear protrusions or atypical nuclei) in epithelial cells of the forestomach and colon even if an increased mitotic index was noticed. In addition, no micronuclei in spermatids were found (Sycheva et al, 2011). Furthermore, TiO_2 P25 NPs orally administrated in mice induced DNA strand breaks and chromosomal damage in bone marrow and/or peripheral blood (erythrocytes), probably due to direct inflammatory effects on circulating innate cells as shown by cytokine expression changes in peripheral blood (Trouiller et al, 2009). This study also showed that in utero exposure to TiO_2 P25 induced DNA deletions in the fetus of exposed pregnant dams.

Two studies used inhalation or intratracheal exposure and both showed negative genotoxicity outcome for the TiO_2 NPs tested (Lindberg et al, 2012; Naya et al, 2012). Inhaled TiO_2 NPs in mice induced inflammatory effects in BAL fluid without significant genotoxic effects locally in lung epithelial cells or systematically in peripheral blood polychromatic erythrocytes (Lindberg et al, 2012). Furthermore, a single intratracheal instillation of TiO_2 anatase NPs or repeated intratracheal instillation, once a week for 5 weeks, induced an inflammatory response, but not DNA damage as assessed by comet assay, in lung tissues of rats (Naya et al, 2012). In contrast to these studies repeated intraperitoneal injection for 5 consecutive days of TiO_2 anatase/rutile NPs resulted in significant increases of titanium content in bone marrow (highest accumulation), liver, and brain cells (lowest accumulation) of mice exposed, resulting in dose-dependent increases of both micronuclei frequency in erythrocytes and DNA damage in mouse bone marrow, liver and brain cells (El-Ghor et al, 2014). Both the observed mutations in *p53* and genotoxicity were normalized after co-administration of the free radical scavenger chlorophyllin suggesting oxidative stress as the possible mechanism for TiO_2 NPs genotoxicity and mutagenicity (El-Ghor et al, 2014).

Taken together, both positive and negative results were reported in the *in vivo* studies on TiO_2 NPs. Both studies with administration via the lung were negative as well as three of four studies using intravenous administration. In contrast, three of four with oral/intragastic studies were positive.

4.2.2 Examples of in vitro studies

Conflicting results in the literature have shown either positive or negative genotoxic effects of TiO_2 NPs in different cell lines (Table IV). TiO_2 anatase NPs induced both chromatid and chromosome aberrations in isolated human lymphocytes whereas TiO_2 P25 NPs (anatase/rutile) induced chromatid breaks, exchanges and polyploidy only after UV irradiation in Chinese hamster lung cells and negative results in human skin fibroblast cells (Catalán et al, 2012; Nakagawa et al, 1997; Browning et al, 2014). In terms of MN induction, TiO_2 anatase NPs enanched MN formation in Chinese hamster ovary cells, human bronchial epithelial lung cells, and lymphoblastoid B-cells (Di Virgilio et al, 2010; Falck et al, 2009; Wang et al, 2007). When the role of crystalline forms of TiO_2 NPs was compared (anatase vs rutile) no conclusive information was obtained. For instance, TiO_2 rutile NPs and not anatase induced MN formation in Balb/3T3 mouse fibroblasts whereas TiO_2 anatase, and not rutile, increased the MN frequency in human bronchial epithelial BEAS-2B cells (Uboldi et al, 2016; Falck et al, 2009). Additionally, both nanosized and bulk TiO_2 rutile particles were able to induce neoplastic effects as evaluated by type-III foci formation in Balb/3T3 cells whereas nanosized and bulk TiO_2 anatase did not (Uboldi et al, 2016). However, despite the increased intracellular ROS levels and higher p53-binding protein 1 foci counts (non-enzymatic protein recruited after double strand breaks or replication fork block), TiO_2 P25 NPs (anatase/rutile) did not induce MN formation following 2-month exposure in lung adenocarcinoma A549 cells whereas both primary and oxidative DNA damage were observed as assessed by comet assay (Armand et al, 2016). The different sensitivity of comet and micronucleus assays could be related to the specific genotoxic mechanisms exerted by TiO_2 NPs. In fact, the relative insensitivity of the in vitro micronucleus assay to agents that predominantly induce adducts and other excision-repairable lesions it is well known (Fenech and Neville, 1992), and the increase of p53-binding protein 1 foci counts involve early DNA damage-signaling pathways that trigger DNA repair (Rappold et al, 2001). However, both comet assay and MN test showed positive results when oxidative stress was induced by TiO_2 P25 NPs in peripheral blood lymphocytes, and the supplementation of the antioxidant N-acetylcysteine inhibited the level of nano-TiO_2-induced genotoxicity (Kang et al, 2008). Furthermore, different cell types

and TiO_2 NPs dispersion methods could result in different outcomes when evaluating the same NPs showing both positive and negative effects (Ursini et al, 2014; Magdolenova et al, 2012; Shi et al, 2010; Franchi et al, 2015). TiO_2 anatase NPs showed significant mutagenic potential in human lymphoblastoid B-cells WIL2-NS and Chinese hamster lung V79-4 fibroblasts but not in Chinese hamster ovary cells as assessed by *Hprt* mutation assay (Wang et al, 2007; Chen et al, 2014; Wang et al, 2011).

4.3 Au

Gold nanoparticles have gained recent attention for potential application like molecular imaging in deep tissues and carriers for drug and gene delivery applications, among others. However, yet little is known about their safety.

4.3.1 In vivo studies

A summary of *in vivo* data is shown in Table V. Of the five studies found in the literature, only one study report negative outcome (Downs et al, 2012), three showed positive genotoxic effects of Au NPs (Cardoso et al, 2014; Cardoso et al, 2014b; Schulz et al, 2012) and one showed positive effects in off-springs of exposed mice, but not in the adult (exposed) mice (Balansky et al, 2013). In the negative study, gold levels in the liver and lung tissues of intravenously exposed rats were significantly increased, although the levels measured were variable among animals, still without showing any genotoxic effect in any of the organs tested (Downs, et al, 2012). In contrast, both acute and chronic intraperitoneal administration in rats of differently sized AuNPs (10 nm and 30 nm, citrate coated) induced DNA damage in blood and liver cells when evaluated by using the comet assay (Cardoso et al, 2014). Furthermore, since it has been shown that AuNPs can cross the blood-brain barrier, as well as accumulate in the brain (e.g. hippocampus, thalamus, hypothalamus, and the cerebral cortex) (Sousa et al, 2010), DNA damage was also evaluated in the cerebral cortex of rats exposed to the same Au NPs (acute and chronic intraperitoneal administration). The results showed DNA damage also in this tissue with higher DNA damage following chronic exposures when compared to the acute (Cardoso et al, 2014b). In another study, genotoxicity following a single intratracheal instillation of Au NPs (2, 20 and 200) in rats was investigated (Schulz et al, 2012). In this study, no MN induction was found in bone marrow cells, but a weak increase in DNA damage in lung cells was shown. The DNA damage was size related in the sense that larger particles induced higher DNA damage, but a high animal-to-animal variation was noticed (Schulz et al, 2012). In another study, transplacental genotoxic and epigenetic effects were noticed in intraperitoneally exposed mice whereas no signs of genotoxicity were found in adult male, female and exposed pregnant mice (Balansky et al, 2013). The administration of 100 nm AuNPs in pregnant mice altered also fetus organs miRNA expression profiles involved in cell proliferation and adhesion, modulation of oncogenes and tumor suppressors among others (Balansky et al, 2013).

Taken together, these studies showed a potential of AuNPs to translocate over biological barriers and to cause genotoxic and epigenetic effects.

4.3.2 Examples of in vitro studies

In vitro studies indicate that AuNPs induce chromosome aberrations, including telomeric damage and aneuploidy, as well as micronuclei formation, DNA strand breaks and oxidative DNA damage in different cell lines (Di Bucchianico et al, 2014; Fraga et al, 2013; Paino et al, 2012; Li et al, 2011; Di Bucchianico et al, 2015) (Table VI). The surface chemistry of AuNPs

seems to play a pivotal role in induced genotoxicity given that AuNPs prepared in water by laser ablation showed no particular signs of cyto- and genotoxicity whereas when AuNPs were produced in pure acetone the observed genotoxicity was induced as a function of the amount of surface contaminants like amorphous carbon and enolate ions (Di Bucchianico et al, 2015). Regardless of the different surface coating, no differences were observed in the uptake of citrate-AuNPs compared with 11-mercaptoundecanoic acid coated AuNPs in human HepG2 liver cells whereas only citrate-capped AuNPs induced DNA lesions without associated cytotoxicity (Fraga et al, 2013).

4.4 CNT

The high aspect ratio of carbon nanotubes (CNTs) makes them similar to asbestos fibers, and therefore, similar pathogenicity has been suggested (Poland et al, 2011). However, several factors including chemical composition, dimensions, mechanical properties, surface modifications as well as the presence of metallic catalysts, seem to be important for their toxicity in general. Presently, a specific for of multi-walled carbon nanotubes (MWCNTs), the so called MWCNT-7, has been classified as “possibly carcinogenic to humans” by IARC, whereas several others including single-walled CNTs (SWCNTs) were not possible to classify.

4.4.1 In vivo studies

Considering that genomic instability is an important factor contributing to the development of human malignancy, evaluation of genotoxic effects of CNTs represents a fundamental topic of investigation and available studies found in the literature are summarized in Table VII. Of the 7 in vivo studies found, 2 report negative findings (Ema et al, 2012; Portman et al, 2012) whereas five report positive effects for one or several of the investigated materials (Kim et al, 2014, Patlolla et al, 2010 and 2015, Shvedova et al, 2014 and Catalán et al, 2016).

MWCNTs did not induce genotoxicity in lung cells of rats intratracheally instilled as a single dose or repeated instillations (once a week for 5 weeks) when evaluated by using the comet assay in lung cells (Ema et al, 2012). All exposures elicited, however, pulmonary inflammation. Similarly, after a 90-day nose-only inhalation exposure of Wistar rats, no genotoxicity was detected locally in lung and distally in bone marrow, liver and kidney cells while lung inflammation was observed (Pothmann et al, 2015). In contrast, genotoxic effects of MWCNTs in lung cells of nose-only 28-day exposed rats were noticed both 0 days and 90-day post-exposure without increasing inflammatory cytokine levels in the bronchoalveolar lavage (BAL) fluid (Kim et al, 2014). Interestingly, in this study MWCNTs were shown to remain in the lung cells 90-day post exposure. A well-designed and well-conducted study assessed inflammatory, fibrogenic, and genotoxic effects of carbon nanofibers, single wall carbon nanotubes (SWCNTs), and asbestos in mice 1 yr after a single pulmonary exposure by pharyngeal aspiration (Shvedova et al, 2014). The study provides clear evidences that SWCNTs, carbon nanofibers, and asbestos persist in the lung and translocate to regional lymphatics eliciting micronuclei formation, nuclear protrusions and pulmonary fibrosis (Shvedova et al, 2014). Interstingly, also an accumulation of K-ras mutations in lungs was noticed, and the increase of mutation induced 1 yr after inhalation of SWCNTs was greater than after SWCNTs aspiration. This observation raises the question whether different *in vivo* exposure methods could result in different NPs dispersion, distribution and genotoxicological responses. When mice were treated with straight or tangled MWCNTs by aspiration or inhalation exposures, agglomerates of both materials were found mainly inside the bronchia when administered by aspiration, while smaller groups of individual fibers were present

mainly in the alveolar lung tissue after inhalation (Catalán et al, 2016). Catalán and co-authors clearly showed that both MWCNTs were able to induce DNA damage in mouse lungs regardless of the exposure methods but only the better dispersed straight MWCNTs induced DNA damage in BAL cells after inhalation but not after pharyngeal aspiration possibly reflecting changes in BAL cell population. Furthermore, neither straight or tangled MWCNTs were able to induce DNA damage or MN in peripheral blood leukocytes or bone marrow erythrocytes highlighting the need to use techniques revealing local effects instead of assessing systemic genotoxicity as for soluble chemicals (Catalán et al, 2016). In contrast, carboxylated-SWCNTs and carboxylated-MWCNTs significantly increased structural chromosomal aberrations and the frequency of micronuclei in bone marrow cells as well as induced DNA damage in mice leukocytes following intraperitoneal injection (once a day for 5 days) in male Swiss–Webster mices (Patlolla et al, 2010; Patlolla et al, 2015). When functionalized and non-functionalized MWCNTs were compared, carboxylated-MWCNTs induced a higher genotoxicity respect to non-functionalized MWCNTs (Patlolla et al, 2010).

Taken together, although both negative and positive studies were found, quite convincing result show DNA damage in lung cells but not in bone marrow or blood cells following CNT administration via the lungs. Genotoxicity in bone marrow or blood cells was, however, found following intraperitoneal injection.

4.4.2 Examples of in vitro studies

The ability of CNTs to induce genotoxic and mutagenic effects was shown in different cell lines as well as their neoplastic transformation potential was investigated (Table VIII). The toxicological effects of nude and chemically functionalized (-NH_2, -OH and –COOH groups) MWCNTs were investigated showing the ability of all types of MWCNTs to induce neoplastic transformation as assessed by CTA in Balb/3T3 mouse fibroblasts (Ponti et al, 2013). Furthermore, the carcinogenic potential of MWCNTs is not triggered by MN induction and not uptake-mediated since –NH_2 and –OH MWCNTs were able to enter into the cells whereas –COOH and nude MWCNTs were not (Ponti et al, 2013). However, both MN induction and cell-transformation effects were showed following sub-chronic (up to 4 weeks) exposures to low doses of MWCNTs in BEAS-2B human bronchial epithelial cells (Vales et al, 2016). Nevertheless, despite intracellular ROS induction (also associated to the metals contaminants present in MWCNTs) no primary DNA damage was noticed by the comet assay. However, when the genotoxic response to 15 MWCNTs with variable physicochemical properties was assessed in the FE1-Muta(TM) mouse lung epithelial cells, increased levels of DNA damage were observed only following exposures to MWCNTs with large diameters and high Fe_2O_3 and Ni content (Jackson et al, 2015). The role of residual catalyst metals in CNTs genotoxicity was suggested as the main responsible factor for the SWCNTs induced genotoxicity in mouse macrophages since microanalysis data revealed that nickel catalyst aggregates both in the culture medium and in the macrophages phagolysosomes (Di Giorgio et al, 2011). Moreover, the increased intracellular ROS suggested that oxidative DNA damage could be the main mechanism of action by which genotoxicity occurs in RAW264.7 macrophages after both SWCNTs and MWCNTs exposure. However, the genotoxicity of MWCNTs was independent of the low amount of metal contaminant in MWCNTs samples and the induced genotoxicity was related to their incomplete phagocytosis or to the damaged plasma membrane (Di Giorgio et al, 2011). Aneuploidogenic effects and intracellular ROS increase induced by MWCNTs were again correlated to mechanical damages in lung carcinoma A549 cells compartments (Visalli et al, 2015). In human bronchial epithelial BEAS-2B cells, straight MWCNTs induced DNA strand breaks at low doses while tangled MWCNTs increased DNA damage only at high concentrations (Catalán et al, 2016).

However, in this study it was shown that neither of the MWCNTs was able to induce micronuclei, but despite the fact that the top doses in the MN assay were chosen in order to avoid interactions with the microscopical analysis of MN, the concurrent cytostasis data was above the upper limit for testing by OECD. Interestingly, MWCNTs induced ROS-mediated *Hprt* mutations in Chinese hamster lung fibroblasts and the mutagenic activity of different SWCNTs mirrored the different levels of oxidative stress induced in human lymphoblastoid MCL-5 B cells (Rubio et al, 2016; Manshiann et al, 2013).

4.5 Ag

Silver NPs (AgNPs) represent an important class of nanomaterials used in many product categories like food, consumer and medical products available in the marketplace mainly due to antimicrobial properties of Ag (Wijnhoven et al, 2009). Although many *in vitro* studies assessed the genotoxic potential of AgNPs, the number of *in vivo* studies is very limited. One main difference in the toxicological profile of AgNPs, when compared to soluble Ag forms, is related to the possible difference in cellular uptake, tissue distribution and subsequent toxic mechanisms. For instance, less than 1% of the initial dose of AgNPs and more than 7% of the initial dose of soluble $AgNO_3$ was recovered in the liver 4 h following intratracheal instillation in mice, suggesting that the ionic form of Ag was absorbed by the lung tissue and entered the systemic circulation more efficiently than AgNPs (Arai et al, 2015). However, intravenously injected AgNPs caused peripheral inflammation and inflammatory infiltrates were revealed by pathological and histological observations in the vessel walls of liver, kidney and lung, whereas $AgNO_3$ did not induce peripheral inflammation suggesting that AgNPs have unique mechanisms of action (Guo et al, 2016).

4.5.1 In vivo studies

The *in vivo* studies of Ag NPs found in the literature are compiled in Table IX. Four studies were found and both positive and negative results were reported for genotoxicity. Although both PVP- and silica coated AgNPs could reach the bone marrow and liver tissues following intravenous injection in mice, no signs of genotoxicity were found in terms of MN induction or mutagenicity in bone marrow cells, and no DNA strand breaks were detected in liver cells (Li et al, 2014). In contrast, all AgNPs induced oxidative DNA damage as evaluated by the modified-comet assay using ENDOIII and hOGG1 enzymes (Li et al, 2014). A dose-dependent increase in Ag was found in the blood, stomach, brain, liver, kidneys, lungs, and testes following 28-days orally exposure of rats, indicating that the AgNPs were systemically distributed, but only a slight and not significant increase of MN in reticulocytes was noticed (Kim et al, 2008). However, following a single intravenous injection, 200 nm sized Ag sub-micro particles but not 20 nm sized AgNPs caused oxidative DNA damage in lung and testis of wild-type mice, as well as DNA double strand breaks in lung of $Ogg1^{-/-}$ mice whereas only 20 nm AgNPs elicited significant primary DNA damage in lung cells of wild-type mice (Asare et al, 2015). Additionally, up-regulation of key genes involved in DNA damage response/repair pathway, in antioxidant response and in immediate-early response was noticed particularly in KO mice. Furthermore, AgNPs induced chromatid breaks and DNA damage in bone marrow cells, with a concurrent ROS increase, following a single intraperitoneal injection in Swiss albino mice (Ghosh et al, 2012).

Despite few available studies, oxidative stress related genotoxicity seems to be the key mechanism of AgNPs toxic potential.

4.5.2 Examples of in vitro studies

According to the reviewed papers AgNPs are clearly genotoxic and mutagenic *in vitro* (Table X). Indeed, most of the studies analyzing MN induction showed positive results and the same was observed for DNA strand breaks and oxidatively damaged DNA. An important consistency was observed for the positive results obtained by MN and comet assays (Souza et al, 2016; Jiang et al, 2013; Kim et al, 2013; Lim et al, 2012; Kim et al, 2011; AshaRani et al, 2009). Interestingly, the analysis of chromosome aberrations in human circulating lymphocytes revealed that AgNPs induce chromatid breaks in concentration-dependent manner and the incidence of chromosomal aberrations does not correlate with the incidence of observed MN induction, highlighting that the increase of micronuclei could be rather consequence of spindle apparatus malfunction than direct breakage effect on cell DNA (Joksić et al, 2016). However, in human adipose-tissue derived mesenchymal stem cells AgNPs were distributed both to the cytoplasm and nucleus inducing DNA damage as assessed by comet assay and chromosomal aberrations (Hackenberg et al, 2011). Ag NPs increased transcript levels of growth arrest and DNA-damage-inducible gene (*GADD45a*), induced the phosphorylation of proteins γH2AX as well as both primary and oxidative DNA damage in untrasformed human fibroblasts (Franchi et al, 2015).

Generally, the mutagenic potential of AgNPs was concurrent with their ability to induce DNA oxidation both in Chinese hamster lung V79-4 fibroblasts and mouse lymphoma cells (Huk et al, 2014; Huk et al, 2015; Mei et al, 2012).

AgNPs genotoxicity is not always size dependent and independent mechanisms for cytotoxicity and DNA damage were also suggested as well as the important role of surface chemical composition was noticed in driving genotoxic and mutagenic mechanisms of AgNPs (Huk et al, 2014; Gliga et al, 2014).

5 Conclusions

In this report, the most common assays for testing genotoxicity of NMs have been described and discussed. In general, the assays can be used but some interferences or drawbacks of the assays have been identified. For the comet assay, a risk for overestimation of the DNA damage has been suggested when high concentrations of reactive NPs are tested *in vitro* due to additional damage formed during the assay performance. Most likely, the NPs that cause additional damage also cause "real" damage and thus the risk for "false positives" seems rather small. Photocatalytically active TiO_2 can, however, form damage if light is not properly avoided during assay performance, possibly leading to false positives. For micronucleus assay treatment with Cytochalasin-B (in order to score MN in once-divided cells) can affect NP-uptake and therefore, delayed co-treatment is recommended. Bacterial cells have limited ability to engulf NPs and thus, mammalian cells are recommended for mutagenicity testing. One important question for all NP studies is dosimetry consideration and the fact that the real cell dose is seldom measured. The *in vivo* genotoxicity SiO2, TiO2, Au, Ag and CNTs showed that following administration via the lung, no effects on blood or bone marrow cells were in general observed. In contrast, local effects in lung cells were observed convincingly for CNTs, but not for the other NPs. For TiO_2, several studies showed positive findings following oral exposure (in blood, bone marrow or colon). Both Au and Ag NPs showed positive finding following injections and convincingly positive genotoxicity in a range of *in vitro* studies. From the *in vivo* studies it is thus apparent that the administration route is important when studying the genotoxicity of NMs and a focus on target tissue (e.g. lung following inhalation and intratracheal administration), is critical.

6 References

Arai Y, Miyayama T, Hirano S. Difference in the toxicity mechanism between ion and nanoparticle forms of silver in the mouse lung and in macrophages. Toxicology. 2015 Feb 3;328:84-92.

Armand L, Tarantini A, Beal D, Biola-Clier M, Bobyk L, Sorieul S, Pernet-Gallay K4, Marie-Desvergne C, Lynch I, Herlin-Boime N, Carriere M. Long-term exposure of A549 cells to titanium dioxide nanoparticles induces DNA damage and sensitizes cells towards genotoxic agents. Nanotoxicology. 2016 Sep;10(7):913-23.

Asare N, Duale N, Slagsvold HH, Lindeman B, Olsen AK, Gromadzka-Ostrowska J, Meczynska-Wielgosz S, Kruszewski M, Brunborg G, Instanes C. Genotoxicity and gene expression modulation of silver and titanium dioxide nanoparticles in mice. Nanotoxicology. 2016 Apr;10(3):312-21.

AshaRani PV, Low Kah Mun G, Hande MP, Valiyaveettil S. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. ACS Nano. 2009 Feb 24;3(2):279-90.

Balansky R, Longobardi M, Ganchev G, Iltcheva M, Nedyalkov N, Atanasov P, Toshkova R, De Flora S, Izzotti A. Transplacental clastogenic and epigenetic effects of gold nanoparticles in mice. Mutat Res. 2013 Nov-Dec;751-752:42-8.

Braakhuis HM, Kloet SK, Kezic S, Kuper F, Park MV, Bellmann S, van der Zande M, Le Gac S, Krystek P, Peters RJ, Rietjens IM, Bouwmeester H. Progress and future of in vitro models to study translocation of nanoparticles. Arch Toxicol. 2015 Sep;89(9):1469-95.

Browning CL, The T, Mason MD, Wise JP Sr. Titanium Dioxide Nanoparticles are not Cytotoxic or Clastogenic in Human Skin Cells. J Environ Anal Toxicol. 2014 Nov;4(6).

Brun E, Barreau F, Veronesi G et al (2014). Titanium dioxide nanoparticle impact and translocation through ex vivo, in vivo and in vitro gut epithelia. Particle Fibre Toxicol.

Cardoso E, Londero E, Ferreira GK, Rezin GT, Zanoni ET, de Souza Notoya F, Leffa DD, Damiani AP, Daumann F, Rohr P, da Silva L, Andrade VM, da Silva Paula MM. Gold nanoparticles induce DNA damage in the blood and liver of rats. Journal of Nanoparticle Research. 2014 16(11):8p.

Cardoso E, Rezin GT, Zanoni ET, de Souza Notoya F, Leffa DD, Damiani AP, Daumann F, Rodriguez JC, Benavides R, da Silva L, Andrade VM, da Silva Paula MM. Acute and chronic administration of gold nanoparticles cause DNA damage in the cerebral cortex of adult rats. Mutat Res. 2014b Aug-Sep;766-767:25-30.

Catalán J, Järventaus H, Vippola M, Savolainen K, Norppa H. Induction of chromosomal aberrations by carbon nanotubes and titanium dioxide nanoparticles in human lymphocytes in vitro. Nanotoxicology. 2012 Dec;6:825-36.

Catalán J, Siivola KM, Nymark P, Lindberg H, Suhonen S, Järventaus H, Koivisto AJ, Moreno C, Vanhala E, Wolff H, Kling KI, Jensen KA, Savolainen K, Norppa H. In vitro and in vivo genotoxic effects of straight versus tangled multi-walled carbon nanotubes. Nanotoxicology. 2016 Aug;10(6):794-806.

Chen Z, Wang Y, Ba T, Li Y, Pu J, Chen T, Song Y, Gu Y, Qian Q, Yang J, Jia G. Genotoxic evaluation of titanium dioxide nanoparticles in vivo and in vitro. Toxicol Lett. 2014 May 2;226(3):314-9.

Chen Z, Wang Y, Ba T, Li Y, Pu J, Chen T, Song Y, Gu Y, Qian Q, Yang J, Jia G. Genotoxic evaluation of titanium dioxide nanoparticles in vivo and in vitro. Toxicol Lett. 2014 May 2;226(3):314-9.

Commission regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European parliament and of the council by establishing a union list of food additives. Off J European Union 2011, 1–177. L 295/1, 12.11.2011.

Corvi R, Aardema MJ, Gribaldo L, Hayashi M, Hoffmann S, Schechtman L, Vanparys P. ECVAM prevalidation study on in vitro cell transformation assays: general outline and conclusions of the study. Mutat Res. 2012 Apr 11;744(1):12-9.

Decan N, Wu D, Williams A, Bernatchez S, Johnston M, Hill M, Halappanavar S. Characterization of in vitro genotoxic, cytotoxic and transcriptomic responses following exposures to amorphous silica of different sizes. Mutat Res Genet Toxicol Environ Mutagen. 2016 Jan 15;796:8-22.

Di Bucchianico S, Fabbrizi MR, Cirillo S, Uboldi C, Gilliland D, Valsami-Jones E, Migliore L. Aneuploidogenic effects and DNA oxidation induced in vitro by differently sized gold nanoparticles. Int J Nanomedicine. 2014 May 8;9:2191-204.

Di Bucchianico S, Migliore L, Marsili P, Vergari C, Giammanco F, Giorgetti E. Cyto- and genotoxicity of Gold nanoparticles obtained by laser ablation in A549 lung adenocarcinoma cells. J Nanopart Res. 2015 17:213.

Di Giorgio ML, Di Bucchianico S, Ragnelli AM, Aimola P, Santucci S, Poma A. Effects of single and multi walled carbon nanotubes on macrophages: cyto and genotoxicity and electron microscopy. Mutat Res. 2011 May 18;722(1):20-31.

Di Virgilio AL, Reigosa M, Arnal PM, Fernández Lorenzo de Mele M. Comparative study of the cytotoxic and genotoxic effects of titanium oxide and aluminium oxide nanoparticles in Chinese hamster ovary (CHO-K1) cells. J Hazard Mater. 2010 May 15;177(1-3):711-8.

Dobrzyńska MM, Gajowik A, Radzikowska J, Lankoff A, Dušinská M, Kruszewski M. Genotoxicity of silver and titanium dioxide nanoparticles in bone marrow cells of rats in vivo. Toxicology. 2014 Jan 6;315:86-91.

Donner EM, Myhre A, Brown SC, Boatman R, Warheit DB. In vivo micronucleus studies with 6 titanium dioxide materials (3 pigment-grade & 3 nanoscale) in orally-exposed rats. Regul Toxicol Pharmacol. 2016 Feb;74:64-74.

Downs TR, Crosby ME, Hu T, Kumar S, Sullivan A, Sarlo K, Reeder B, Lynch M, Wagner M, Mills T, Pfuhler S. Silica nanoparticles administered at the maximum tolerated dose induce genotoxic effects through an inflammatory reaction while gold nanoparticles do not. Mutat Res. 2012 Jun 14;745(1-2):38-50.

El-Ghor AA, Noshy MM, Galal A, Mohamed HR. Normalization of nano-sized TiO2-induced clastogenicity, genotoxicity and mutagenicity by chlorophyllin administration in mice brain, liver, and bone marrow cells. Toxicol Sci. 2014 Nov;142(1):21-32.

Ema M, Masumori S, Kobayashi N, Naya M, Endoh S, Maru J, Hosoi M, Uno F, Nakajima M, Hayashi M, Nakanishi J. In vivo comet assay of multi-walled carbon nanotubes using lung cells of rats intratracheally instilled. J Appl Toxicol. 2013 Oct;33(10):1053-60.

ENV/JM/MONO(2016)1. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO (2016) 1&docLanguage=En

Falck GC, Lindberg HK, Suhonen S, Vippola M, Vanhala E, Catalán J, Savolainen K, Norppa H. Genotoxic effects of nanosized and fine TiO2. Hum Exp Toxicol. 2009 Jun;28(6-7):339-52.

Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis. 2011 Jan;26(1):125-32.

Fenech M, Neville S. Conversion of excision-repairable DNA lesions to micronuclei within one cell cycle in human lymphocytes. Environ Mol Mutagen. 1992;19(1):27-36.

Fraga S, Faria H, Soares ME, Duarte JA, Soares L, Pereira E, Costa-Pereira C, Teixeira JP, de Lourdes Bastos M, Carmo H. Influence of the surface coating on the cytotoxicity, genotoxicity and uptake of gold nanoparticles in human HepG2 cells. J Appl Toxicol. 2013 Oct;33(10):1111-9.

Franchi LP, Manshian BB, de Souza TA, Soenen SJ, Matsubara EY, Rosolen JM, Takahashi CS. Cyto- and genotoxic effects of metallic nanoparticles in untransformed human fibroblast. Toxicol In Vitro. 2015 Oct;29(7):1319-31.

Galloway SM, Aardema MJ, Ishidate M Jr, Ivett JL, Kirkland DJ, Morita T, Mosesso P, Sofuni T. Report from working group on in vitro tests for chromosomal aberrations. Mutat Res. 1994 Jun;312(3):241-61.

Geraets L, Oomen AG, Krystek P, Jacobsen NR, Wallin H, Laurentie M, Verharen HW, Brandon EF, de Jong WH. Tissue distribution and elimination after oral and intravenous administration of different titanium dioxide nanoparticles in rats. Part Fibre Toxicol. 2014 Jul 3;11:30.

Ghosh M, J M, Sinha S, Chakraborty A, Mallick SK, Bandyopadhyay M, Mukherjee A. In vitro and in vivo genotoxicity of silver nanoparticles. Mutat Res. 2012 Dec 12;749(1-2):60-9.

Gliga AR, Skoglund S, Wallinder IO, Fadeel B, Karlsson HL. Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release. Part Fibre Toxicol. 2014 Feb 17;11:11.

Gonzalez L, Lukamowicz-Rajska M, Thomassen LC, Kirschhock CE, Leyns L, Lison D, Martens JA, Elhajouji A, Kirsch-Volders M. Co-assessment of cell cycle and micronucleus frequencies demonstrates the influence of serum on the in vitro genotoxic response to amorphous monodisperse silica nanoparticles of varying sizes. Nanotoxicology. 2014 Dec;8(8):876-84.

Gonzalez L, Sanderson BJ, Kirsch-Volders M. Adaptations of the in vitro MN assay for the genotoxicity assessment of nanomaterials. Mutagenesis. 2011 Jan;26(1):185-91.

Gonzalez L, Thomassen LC, Plas G, Rabolli V, Napierska D, Decordier I, Roelants M, Hoet PH, Kirschhock CE, Martens JA, Lison D, Kirsch-Volders M. Exploring the aneugenic and clastogenic potential in the nanosize range: A549 human lung carcinoma cells and amorphous monodisperse silica nanoparticles as models. Nanotoxicology. 2010 Dec;4:382-95.

Guichard Y, Maire MA, Sébillaud S, Fontana C, Langlais C, Micillino JC, Darne C, Roszak J, Stępnik M, Fessard V, Binet S, Gaté L. Genotoxicity of synthetic amorphous silica nanoparticles in rats following short-term exposure. Part 2: intratracheal instillation and intravenous injection. Environ Mol Mutagen. 2015 Mar;56(2):228-44.

Guidi P, Nigro M, Bernardeschi M, Lucchesi P, Scarcelli V, Frenzilli G. Does the crystal habit modulate the genotoxic potential of silica particles? A cytogenetic evaluation in human and murine cell lines. Mutat Res Genet Toxicol Environ Mutagen. 2015 Oct;792:46-52.

Guidi P, Nigro M, Bernardeschi M, Scarcelli V, Lucchesi P, Onida B, Mortera R, Frenzilli G. Genotoxicity of amorphous silica particles with different structure and dimension in human and murine cell lines. Mutagenesis. 2013 Mar;28(2):171-80.

Guo H, Zhang J, Boudreau M, Meng J, Yin JJ, Liu J, Xu H. Intravenous administration of silver nanoparticles causes organ toxicity through intracellular ROS-related loss of inter-endothelial junction. Part Fibre Toxicol. 2016 Apr 29;13:21.

Hackenberg S, Scherzed A, Kessler M, Hummel S, Technau A, Froelich K, Ginzkey C, Koehler C, Hagen R, Kleinsasser N. Silver nanoparticles: evaluation of DNA damage, toxicity and functional impairment in human mesenchymal stem cells. Toxicol Lett. 2011 Feb 25;201(1):27-33.

Huk A, Izak-Nau E, El Yamani N, Uggerud H, Vadset M, Zasonska B, Duschl A, Dusinska M. Impact of nanosilver on various DNA lesions and HPRT gene mutations - effects of charge and surface coating. Part Fibre Toxicol. 2015 Jul 24;12:25.

Huk A, Izak-Nau E, Reidy B, Boyles M, Duschl A, Lynch I, Dušinska M. Is the toxic potential of nanosilver dependent on its size? Part Fibre Toxicol. 2014 Dec 3;11:65.

IARC Monographs on the evaluation of Carcinogenic Risks to Humans. Vol. 68; 1997.

IARC Monographs on the evaluation of Carcinogenic Risks to Humans. Vol. 93, 2010.

IARC monographs on the evaluation of carcinogenic risks to humans. Sup 7, 49, volume 100C; 2012.

Jackson P, Kling K, Jensen KA, Clausen PA, Madsen AM, Wallin H, Vogel U. Characterization of genotoxic response to 15 multiwalled carbon nanotubes with variable physicochemical properties including surface functionalizations in the FE1-Muta(TM) mouse lung epithelial cell line. Environ Mol Mutagen. 2015 Mar;56(2):183-203.

Jiang X, Foldbjerg R, Miclaus T, Wang L, Singh R, Hayashi Y, Sutherland D, Chen C, Autrup H, Beer C. Multi-platform genotoxicity analysis of silver nanoparticles in the model cell line CHO-K1. Toxicol Lett. 2013 Sep 12;222(1):55-63.

Johnson GE. Mammalian cell HPRT gene mutation assay: test methods. Methods Mol Biol. 2012;817:55-67.

Joksić G, Stašić J, Filipović J, Šobot AV, Trtica M. Size of silver nanoparticles determines proliferation ability of human circulating lymphocytes in vitro. Toxicol Lett. 2016 Apr 15;247:29-34.

Kang GS, Gillespie PA, Gunnison A, Moreira AL, Tchou-Wong KM, Chen LC. Long-term inhalation exposure to nickel nanoparticles exacerbated atherosclerosis in a susceptible mouse model. Environ Health Perspect. 2011 Feb;119(2):176-81.

Kang GS, Gillespie PA, Gunnison A, Rengifo H, Koberstein J, Chen LC. Comparative pulmonary toxicity of inhaled nickel nanoparticles; role of deposited dose and solubility. Inhal Toxicol. 2011b Feb;23(2):95-103.

Kang SJ, Kim BM, Lee YJ, Chung HW. Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. Environ Mol Mutagen. 2008 Jun;49(5):399-405.

Karimi M, Ghasemi A, Sahandi Zangabad P, Rahighi R, Moosavi Basri SM, Mirshekari H, Amiri M, Shafaei Pishabad Z, Aslani A, Bozorgomid M, Ghosh D, Beyzavi A, Vaseghi A, Aref AR, Haghani L, Bahrami S, Hamblin MR. Smart micro/nanoparticles in stimulus-responsive drug/gene delivery systems. Chem Soc Rev. 2016 Mar 7;45(5):1457-501.

Kim HR, Kim MJ, Lee SY, Oh SM, Chung KH. Genotoxic effects of silver nanoparticles stimulated by oxidative stress in human normal bronchial epithelial (BEAS-2B) cells. Mutat Res. 2011 Dec 24;726(2):129-35.

Kim HR, Park YJ, Shin da Y, Oh SM, Chung KH. Appropriate in vitro methods for genotoxicity testing of silver nanoparticles. Environ Health Toxicol. 2013;28:e2013003.

Kim JS, Sung JH, Choi BG, Ryu HY, Song KS, Shin JH, Lee JS, Hwang JH, Lee JH, Lee GH, Jeon K, Ahn KH, Yu IJ. In vivo genotoxicity evaluation of lung cells from Fischer 344 rats following 28 days of inhalation exposure to MWCNTs, plus 28 days and 90 days post-exposure. Inhal Toxicol. 2014 Mar;26(4):222-34.

Kim YS, Kim JS, Cho HS, Rha DS, Kim JM, Park JD, Choi BS, Lim R, Chang HK, Chung YH, Kwon IH, Jeong J, Han BS, Yu IJ. Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhal Toxicol. 2008 Apr;20(6):575-83.

Li JJ, Lo SL, Ng CT, Gurung RL, Hartono D, Hande MP, Ong CN, Bay BH, Yung LY. Genomic instability of gold nanoparticle treated human lung fibroblast cells. Biomaterials. 2011 Aug;32(23):5515-23.

Li Y, Bhalli JA, Ding W, Yan J, Pearce MG, Sadiq R, Cunningham CK, Jones MY, Monroe WA, Howard PC, Zhou T, Chen T. Cytotoxicity and genotoxicity assessment of silver nanoparticles in mouse. Nanotoxicology. 2014 Aug;8 Suppl 1:36-45.

Lim HK, Asharani PV, Hande MP. Enhanced genotoxicity of silver nanoparticles in DNA repair deficient Mammalian cells. Front Genet. 2012 Jun 13;3:104.

Lindberg HK, Falck GC, Catalán J, Koivisto AJ, Suhonen S, Järventaus H, Rossi EM, Nykäsenoja H, Peltonen Y, Moreno C, Alenius H, Tuomi T, Savolainen KM, Norppa H. Genotoxicity of inhaled nanosized TiO(2) in mice. Mutat Res. 2012 Jun 14;745(1-2):58-64.

Lindberg HK, Falck GC, Singh R, Suhonen S, Järventaus H, Vanhala E, Catalán J, Farmer PB, Savolainen KM, Norppa H. Genotoxicity of short single-wall and multi-wall carbon nanotubes in human bronchial epithelial and mesothelial cells in vitro. Toxicology. 2013 Nov 8;313(1):24-37.

Liu Y, Xia Q, Liu Y, Zhang S, Cheng F, Zhong Z, Wang L, Li H, Xiao K. Genotoxicity assessment of magnetic iron oxide nanoparticles with different particle sizes and surface coatings. Nanotechnology. 2014 Oct 24;25(42):425101.

Louro H, Tavares A, Vital N, Costa PM, Alverca E, Zwart E, de Jong WH, Fessard V, Lavinha J, Silva MJ. Integrated approach to the in vivo genotoxic effects of a titanium dioxide nanomaterial using LacZ plasmid-based transgenic mice. Environ Mol Mutagen. 2014 Jul;55(6):500-9.

MacNicoll A, Kelly M, Aksoy H, Kramer E, Bouwmeester H, Chaudhry Q (2015). A study of the uptake and biodistribution of nano-titanium dioxide using in vitro and in vivo models of oral intake. J Nanopart Res 17(66):20.

Magaye R, Zhao J. Recent progress in studies of metallic nickel and nickel-based nanoparticles' genotoxicity and carcinogenicity. Environ Toxicol Pharmacol. 2012 Nov;34(3):644-50.

Magdolenova Z, Bilaničová D, Pojana G, Fjellsbø LM, Hudecova A, Hasplova K, Marcomini A, Dusinska M. Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity. J Environ Monit. 2012 Feb;14(2):455-64.

Magdolenova Z, Lorenzo Y, Collins A, Dusinska M. Can standard genotoxicity tests be applied to nanoparticles? J Toxicol Environ Health A. 2012;75(13-15):800-6.

Manshian BB, Jenkins GJ, Williams PM, Wright C, Barron AR, Brown AP, Hondow N, Dunstan PR, Rickman R, Brady K, Doak SH. Single-walled carbon nanotubes: differential genotoxic potential associated with physico-chemical properties. Nanotoxicology. 2013 Mar;7(2):144-56.

Maser E, Schulz M, Sauer UG, Wiemann M, Ma-Hock L, Wohlleben W, Hartwig A, Landsiedel R. In vitro and in vivo genotoxicity investigations of differently sized amorphous SiO2 nanomaterials. Mutat Res Genet Toxicol Environ Mutagen. 2015 Dec;794:57-74.

Mei N, Zhang Y, Chen Y, Guo X, Ding W, Ali SF, Biris AS, Rice P, Moore MM, Chen T. Silver nanoparticle-induced mutations and oxidative stress in mouse lymphoma cells. Environ Mol Mutagen. 2012 Jul;53(6):409-19.

Mosesso P1, Cinelli S, Natarajan AT, Palitti F. In vitro cytogenetic assays: chromosomal aberrations and micronucleus tests. Methods Mol Biol. 2013;1044:123-46.

Nakagawa Y, Wakuri S, Sakamoto K, Tanaka N. The photogenotoxicity of titanium dioxide particles. Mutat Res. 1997 Nov 27;394(1-3):125-32.

Naya M, Kobayashi N, Ema M, Kasamoto S, Fukumuro M, Takami S, Nakajima M, Hayashi M, Nakanishi J. In vivo genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats. Regul Toxicol Pharmacol. 2012 Feb;62(1):1-6.

OECD 451. Carcinogenicity Studies. OECD Publishing, Paris.

OECD 473. In vitro Mammalian Chromosome Aberration Test. OECD Publishing, Paris.

OECD 474. Mammalian Erythrocyte Micronucleus Test. OECD Publishing, Paris.

OECD 475. Mammalian Bone Marrow Chromosome Aberration Test. OECD Publishing, Paris.

OECD 484. Genetic toxicology: mouse spot test. OECD Publishing, Paris.

OECD 487. In Vitro Mammalian Cell Micronucleus Test. OECD Publishing, Paris.

Paino IM, Marangoni VS, de Oliveira Rde C, Antunes LM, Zucolotto V. Cyto and genotoxicity of gold nanoparticles in human hepatocellular carcinoma and peripheral blood mononuclear cells. Toxicol Lett. 2012 Nov 30;215(2):119-25.

Pan X, Redding JE, Wiley PA, Wen L, McConnell JS, Zhang B. Mutagenicity evaluation of metal oxide nanoparticles by the bacterial reverse mutation assay. Chemosphere. 2010 Mar;79(1):113-6.

Papageorgiou I, Brown C, Schins R, Singh S, Newson R, Davis S, Fisher J, Ingham E, Case CP. The effect of nano- and micron-sized particles of cobalt-chromium alloy on human fibroblasts in vitro. Biomaterials. 2007 Jul;28(19):2946-58.

Patlolla AK, Hussain SM, Schlager JJ, Patlolla S, Tchounwou PB. Comparative study of the clastogenicity of functionalized and nonfunctionalized multiwalled carbon nanotubes in bone marrow cells of Swiss-Webster mice. Environ Toxicol. 2010 Dec;25(6):608-21.

Patlolla AK, Patra PK, Flountan M, Tchounwou PB. Cytogenetic evaluation of functionalized single-walled carbon nanotube in mice bone marrow cells. Environ Toxicol. 2015 Feb 17. doi: 10.1002/tox.22118. [Epub ahead of print]

Pfuhler S, Elespuru R, Aardema MJ, Doak SH, Maria Donner E, Honma M, Kirsch-Volders M, Landsiedel R, Manjanatha M, Singer T, Kim JH. Genotoxicity of nanomaterials: refining strategies and tests for hazard identification. Environ Mol Mutagen. 2013 May;54(4):229-39.

Ponti J, Broggi F, Mariani V, De Marzi L, Colognato R, Marmorato P, Gioria S, Gilliland D, Pascual Garcìa C, Meschini S, Stringaro A, Molinari A, Rauscher H, Rossi F. Morphological transformation induced by multiwall carbon nanotubes on Balb/3T3 cell model as an in vitro end point of carcinogenic potential. Nanotoxicology. 2013 Mar;7(2):221-33.

Pothmann D, Simar S, Schuler D, Dony E, Gaering S, Le Net JL, Okazaki Y, Chabagno JM, Bessibes C, Beausoleil J, Nesslany F, Régnier JF. Lung inflammation and lack of genotoxicity in the

comet and micronucleus assays of industrial multiwalled carbon nanotubes Graphistrength(©) C100 after a 90-day nose-only inhalation exposure of rats. Part Fibre Toxicol. 2015 Jul 10;12:21.

Rappold I, Iwabuchi K, Date T, Chen J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. J Cell Biol. 2001 Apr 30;153(3):613-20.

Rubio L, El Yamani N, Kazimirova A, Dusinska M, Marcos R. Multi-walled carbon nanotubes (NM401) induce ROS-mediated HPRT mutations in Chinese hamster lung fibroblasts. Environ Res. 2016 Apr;146:185-90.

Sadiq R, Bhalli JA, Yan J, Woodruff RS, Pearce MG, Li Y, Mustafa T, Watanabe F, Pack LM, Biris AS, Khan QM, Chen T. Genotoxicity of TiO(2) anatase nanoparticles in B6C3F1 male mice evaluated using Pig-a and flow cytometric micronucleus assays. Mutat Res. 2012 Jun 14;745(1-2):65-72.

Sargent LM, Reynolds SH, Castranova V. Potential pulmonary effects of engineered carbon nanotubes: in vitro genotoxic effects. Nanotoxicology. 2010 Dec;4:396-408.

Schulz M, Ma-Hock L, Brill S, Strauss V, Treumann S, Gröters S, van Ravenzwaay B, Landsiedel R. Investigation on the genotoxicity of different sizes of gold nanoparticles administered to the lungs of rats. Mutat Res. 2012 Jun 14;745(1-2):51-7.

Shi Y, Zhang JH, Jiang M, Zhu LH, Tan HQ, Lu B. Synergistic genotoxicity caused by low concentration of titanium dioxide nanoparticles and p,p'-DDT in human hepatocytes. Environ Mol Mutagen. 2010 Apr;51(3):192-204.

Shoemaker RH, Wolpert-DeFilippes MK, Kern DH, Lieber MM, Makuch RW, Melnick NR, Miller WT, Salmon SE, Simon RM, Venditti JM, et al. Application of a human tumor colony-forming assay to new drug screening. Cancer Res. 1985 May;45(5):2145-53.

Shvedova AA, Yanamala N, Kisin ER, Tkach AV, Murray AR, Hubbs A, Chirila MM, Keohavong P, Sycheva LP, Kagan VE, Castranova V. 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 Jan;306(2):L170-82.

Sousa F, Mandal S, Garrovo C, Astolfo A, Bonifacio A, Latawiec D, Menk RH, Arfelli F, Huewel S, Legname G, Galla HJ, Krol S. Functionalized gold nanoparticles: a detailed in vivo multimodal microscopic brain distribution study. Nanoscale. 2010 Dec;2(12):2826-34.

Souza TA, Franchi LP, Rosa LR, da Veiga MA, Takahashi CS. Cytotoxicity and genotoxicity of silver nanoparticles of different sizes in CHO-K1 and CHO-XRS5 cell lines. Mutat Res Genet Toxicol Environ Mutagen. 2016 Jan 1;795:70-83.

Stark WJ, Stoessel PR, Wohlleben W, Hafner A. Industrial applications of nanoparticles. Chem Soc Rev. 2015 Aug 21;44(16):5793-805. Review.

Suzuki T, Miura N, Hojo R, Yanagiba Y, Suda M, Hasegawa T, Miyagawa M, Wang RS. Genotoxicity assessment of intravenously injected titanium dioxide nanoparticles in gpt delta transgenic mice. Mutat Res Genet Toxicol Environ Mutagen. 2016 May;802:30-7.

Sycheva LP, Zhurkov VS, Iurchenko VV, Daugel-Dauge NO, Kovalenko MA, Krivtsova EK, Durnev AD. Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice in vivo. Mutat Res. 2011 Nov 27;726(1):8-14.

Tanaka N, Bohnenberger S, Kunkelmann T, Munaro B, Ponti J, Poth A, Sabbioni E, Sakai A, Salovaara S, Sasaki K, Thomas BC, Umeda M. Prevalidation study of the BALB/c 3T3 cell transformation assay for assessment of carcinogenic potential of chemicals. Mutat Res. 2012 Apr 11;744(1):20-9.

Tarantini A, Huet S, Jarry G, Lanceleur R, Poul M, Tavares A, Vital N, Louro H, João Silva M, Fessard V. Genotoxicity of synthetic amorphous silica nanoparticles in rats following short-term exposure. Part 1: oral route. Environ Mol Mutagen. 2015 Mar;56(2):218-27.

Tice RR, Hayashi M, MacGregor JT, Anderson D, Blakey DH, Holden HE, Kirsch-Volders M, Oleson FB Jr, Pacchierotti F, Preston RJ, et al. Report from the working group on the in vivo mammalian bone marrow chromosomal aberration test. Mutat Res. 1994 Jun;312(3):305-12.

Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Res. 2009 Nov 15;69(22):8784-9.

Uboldi C, Urbán P, Gilliland D, Bajak E, Valsami-Jones E, Ponti J, Rossi F. Role of the crystalline form of titanium dioxide nanoparticles: Rutile, and not anatase, induces toxic effects in Balb/3T3 mouse fibroblasts. Toxicol In Vitro. 2016 Mar;31:137-45.

Ursini CL, Cavallo D, Fresegna AM, Ciervo A, Maiello R, Tassone P, Buresti G, Casciardi S, Iavicoli S. Evaluation of cytotoxic, genotoxic and inflammatory response in human alveolar and bronchial epithelial cells exposed to titanium dioxide nanoparticles. J Appl Toxicol. 2014 Nov;34(11):1209-19.

Vales G, Rubio L, Marcos R. Genotoxic and cell-transformation effects of multi-walled carbon nanotubes (MWCNT) following in vitro sub-chronic exposures. J Hazard Mater. 2016 Apr 5;306:193-202.

van der Zande M, Vandebriel RJ, Groot MJ et al (2014). Sub-chronic toxicity study in rats orally exposed to nanostructured silica. Part Fibre Toxicol 11:8.

Visalli G, Bertuccio MP, Iannazzo D, Piperno A, Pistone A, Di Pietro A. Toxicological assessment of multi-walled carbon nanotubes on A549 human lung epithelial cells. Toxicol In Vitro. 2015 Mar;29(2):352-62.

Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, Jia G, Gao Y, Li B, Sun J, Li Y, Jiao F, Zhao Y, Chai Z. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol Lett. 2007 Jan 30;168(2):176-85.

Wang JJ, Sanderson BJ, Wang H. Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells. Mutat Res. 2007 Apr 2;628(2):99-106.

Wang JJ, Sanderson BJ, Wang H. Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells. Mutat Res. 2007 Apr 2;628(2):99-106.

Wang S, Hunter LA, Arslan Z, Wilkerson MG, Wickliffe JK. Chronic exposure to nanosized, anatase titanium dioxide is not cyto- or genotoxic to Chinese hamster ovary cells. Environ Mol Mutagen. 2011 Oct;52(8):614-22.

Wijnhoven SWP, Peijnenburg WJGM, Herberts CA, et al. Nano-silver - A review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology. 2009 June;3(2):109-38.

7 Tables

7.1.1 Table I. In vivo SiO_2

Nanoparticle	Primary particle size (nm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
NM-200 NM-201 NM-202 NM-203	18.3 ± 4.5 18.0 ± 1.0 17.7 ± 2.5 24.7 ± 17.7	Male Sprague-Dawley rats	Intratracheal instillation and intravenous injection	9, 18 and 36 mg/Kg (instill.); 15, 30, 60 mg/Kg (inject.)	3, 24 and 48 hrs	Comet assay, Fpg-comet and bone marrow micronucleus assay	All particles caused lung inflammation without any significant DNA damage	Guichard 2015
NM-200 NM-201 NM-202 NM-203	18.3 ± 4.5 18.0 ± 1.0 17.7 ± 2.5 24.7 ± 17.7	Male Sprague-Dawley rats	oral exposure	5, 10, or 20 mg/Kg b.w./day	3 days	Comet assay, Fpg-comet, bone marrow and colon micronucleus assays	Weak but significant MN increase in the colon of rats treated with NM-202 and NM-203 at the lowest dose	Tarantini 2015
Levasil® 200/40%	15	Adult male Wistar rats	Intravenous injection	25–50 mg/kg	4, 24, 48 hrs	Comet assay (blood, liver and lung tissues), MN-reticulocytes assay by flow cytometer	Small but reproducible increase in DNA damage and micronucleated reticulocytes when tested at their maximum tolerated dose	Downs 2012
Levasil® 50/50%	55			25–125 mg/kg				
SiO_2	15 55	Wistar Hannover rats	Intratracheal instillation	360 µg	3 days	Comet assay (lung tissues), MN-reticulocytes assay	All particles caused lung inflammation without any significant DNA damage	Maser 2015

7.1.2 Table II. In vitro SiO_2

NPs	Primary Size (nm)	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Notes	Reference
SiO2	12	12.5	12	Mouse lung epithelial cells (FE1)	(+)	(+)	NA	NA	(-)*	*, transgene *lac* Z mutant frequency. Micron-sized particle exhibited the highest potential to induce oxidative stress compared to the SiO2 NPs.	Decan 2016
	5-10				(+)	(+)					
	10-15				(+)	(+)					
	2 µm				(-)	(-)					
Stöber silica nanoparticles	16.4±2.5	10-60 (MN), 40-60 (comet)	40 (MN), 15 min and 4 (comet)	Human lung epithelial cells (A549)	(-)	(-)	(-)	(-)	NA	A slight increase of aneuploidogenic events was assessed by pan-centromeric FISH analysis.	Gonzalez 2010
	60.4±8.3						(-)	(-)			
	104±9.9	40-300 µg/mL					NA	NA			
Lysine catalysed synthesis (L-12)	12	0.025-0.15	40	Human lung epithelial cells (A549)	(+)	(-)	NA	NA	NA	L-40, S-59, S-139, S-174 particles induced G1 and S phase arrest in absence of serum	Gonzalez 2014
		25-125			(+)	(-)					
Stöber silica (S-28)	28	2.5-32.5			(+)	(-)					
		25-134			(-)	(-)					
Lysine catalysed synthesis (L-40)	40	0.05-12.5			(+)	(+)					
		125-1056			(-)	(-)					
Stöber silica (S-59)	59	12.5-100			(+)	(+)					
		25-173			(-)	(-)					
Stöber silica (S-139)	139	1.5-100			(+)	(-)					
		250-510			(-)	(-)					
Stöber silica (S-174)	174	125-200			(-)	(-)					
		50-211			(-)	(-)					
quartz	0.5-5 µm	5-80 µg/cm^2	48 (MN), 4 and 24 (comet)	Human lung epithelial cells (A549)	(+)	(-)	(-)	NA	NA	the different susceptibilities of RAW264.7and A549 cells to silica cannot be ascribed to different uptake	Guidi 2015
amorphous SiO2					(-)	(-)	(-)				
quartz				murine macrophages (Raw264.7)	(+)	(-)	(+)				
amorphous SiO2					(-)	(-)	(-)				

NPs	Primary Size (nm)	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Notes	Reference
Dense silica	250	5-80 µg/cm^2	48 (MN), 4 and 24 (comet)	Human lung epithelial cells (A549)	(-)	(-)	(+)	NA	NA	*, as eavaluated at 10 or 20 µg/cm^2 due to cytotoxicity at highest concentrations	Guidi 2013
	500					(-)	(+)				
Mesoporous silica	250					(-)	(+)				
	500					(-)	(+)				
Dense silica	250			murine macrophages (Raw264.7)	(-)	(+)	(+)				
	500				(+)	(-)*	(+)				
Mesoporous silica	250				(+)	(-)*	(+)				
	500				(+)	(-)*	(+)				
Levasil® 200/40%	15	4-64	24	human colon Caco-2-HTB-37™ cells	(-)	(+)	NA	NA	NA	The observed genotoxic effects are mediated through oxidative stress rather than a direct interaction with the DNA	Tarantini 2015
Levasil® 50/50%	55					(-)					
Quartz DQ12	200					(-)					
Pyrogenic 20	19±5	12.5-100 µg/cm^2	24, 3 and 24 (comet)	Chinese hamster lung fibroblasts (V79-4)	(++)	(-)	(++)	(++)	(-)	The influence of particle agglomeration and oxidative species formation is discussed	Guichard 2015
Pirogenic 25/70	25±8, 71±25				(+)	(-)	(-)	(-)	(-)		
Precipitated 20	19±3				(++)	(-)	(-)	(-)	(-)		
Colloidal 15	15±4				(+++)	(-)	(+)	(+)	(-)		
Colloidal 40/80	78±3, 38±5				(+)	(-)	(-)	(-)	(-)		

7.1.3 Table III. In vivo TiO_2

Nanoparticle	Primary particle size (nm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
TiO_2 anatase	75± 15	Sprague-Dawley male rats	intragastric administration	10, 50 and 200 mg/Kg	every day for 30 days	bone marrow micronucleus assay and γ-H_2AX assay	TiO_2 NPs induced DNA double strand breaks in bone marrow cells without chromosomes or mitotic apparatus damage.	Chen 2014
TiO_2 anatase/rutile NM-105	21	male Wistar rats	single intravenous injection	5 mg/Kg	24 h, 1 week and 4 weeks	bone marrow comet assay (leukocytes) and MN assay (reticulocytes and erythrocytes)	TiO_2 NPs did not induce MN in reticulocytes and DNA strand breaks in leukocytes whereas a significant increase of MN frequency in bone marrow polychromatic erythrocytes was shown 24 h post exposure.	Dobrzyńska 2014
TiO_2 anatase/rutile	43	male and female male Wistar rats	oral gavage	500, 1000 and 2000 mg/Kg	24 and 72 h	bone marrow MN assay in peripheral blood reticulocytes	No genotoxicity without absorption of TiO2 NPs from the gastrointestinal tract into the blood circulation.	Donner 2016
TiO2 anatase	42							
TiO_2 rutile	47							
TiO_2 anatase	153							
TiO_2 rutile	195							
TiO_2 rutile	213							
TiO_2 anatase/brookite	84.5	male C57BL/6J mice	inhalation exposures	0.8 mg/m^3	4 h per day during 5 days	MN assay (erythocites) and Comet assay (alveolar and Clara cells)	No genotoxicity although the highest exposure level produced inflammatory effects.	Lindberg 2012
	73.9			7.2 mg/m^3				
	89.2			28.5 mg/m^3				

Nanoparticle	Primary particle size (nm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
TiO_2 anatase	5	male Crl: CD (SD) rats	single intratracheal instillation repeated intratracheal instillation	1 or 5 mg/kg 0.2 or 1 mg/Kg	3 and 24 h once a week for 5 weeks	Comet assay (lung tissues)	Both treatment conditions induced an inflammatory response, but not DNA damage.	Naya 2012
TiO_2 anatase	12.1± 3.2	male B6C3F1 mice	repeated intravenous injection	0.5, 5 and 50 mg/Kg/day	3 days	bone marrow MN assay in peripheral blood reticulocytes and Pig-a assay	TiO2 NPs are not genotoxic when assessed with in vivo micronucleus or mutagenic when assessed with Pig-a gene mutation tests.	Sadiq 2012
TiO_2 P25	150 (in suspension)	male C57BL/6J gpt Delta mice	repeated intravenous injection	2, 10 and 50 mg/Kg	once a week for 4 weeks	bone marrow MN assay in peripheral blood reticulocytes, comet assay, gpt and Spi^- mutation assays (liver tissues)	TiO2 NPs were not genotoxic or mutagenic.	Suzuki 2016

Nanoparticle	Primary particle size (nm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
TiO_2 anatase	160± 59.4 and 33.2± 16.7	male CBAxB6 mice	oral gavage	40, 200, 1000 mg/Kg/day	1 week	Comet assay (bone marrow, brain, liver cells), bone marrow MN assay (erythrocytes), poly-organ karyological assay	Both particles induced cytogenetic effects probably caused by secondary genotoxic mechanism associated with inflammation and/or oxidative stress.	Sycheva 2011
TiO_2 anatase/rutile	<100	male Swiss Webster mice	repeated intraperitoneal injection	500, 1000, 2000 mg/Kg	once a day for 5 days	Comet assay (bone marrow, brain, liver cells) and bone marrow MN assay (erythrocytes)	Dose-dependent genotoxicity of TiO_2 NPs indicated by the significant increase of both micronuclei frequency and DNA damage. Co-administration of chlorophyllin reduced the induced genotoxicity.	El-Ghor 2014
TiO_2 P25	21	C57Bl/6Jp^{un}/p^{un} mice	exposure in drinking water	500 mg/Kg	5 days	Comet assay (peripheral blood) and MN assay (erythrocytes) and γ-H2AX assay	TiO_2 NPs induced DNA strand breaks and micronuclei as well as increased the frequency of DNA lesions in the fetus of exposed pregnant dams.	Trouiller 2009

Nanoparticle	Primary particle size (nm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
TiO2 anatase (NM-102)	22	LacZ Plasmid-based transgenic C57B1/6 mice	intravenous injection	10 or 15 mg/Kg	2 days	MN assay (reticulocytes) after 48 h post-exp. Comet assay and LacZ mutation assay (liver and spleen cells) 28 days after post-exp.	No genotoxicity despite the TiO_2 NPs biopersistence and a moderate inflammation in the liver 28 days post-exposure.	**Louro 2014**

7.1.4 Table IV. In vitro TiO_2

NPs	Primary Size (nm)	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Chromosome aberrations	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Cell transformation	Reference
TiO2 - Anatase	<25	6.25-300	24-48-72	Isolated human lymphocytes	(-)	(+, 24 and 48 h)	NA	NA	NA	NA	NA	Catalán 2012
TiO2 P-25 (anatase:rutile 8:2)	<25	3-800	1 h w/ or w/o UV	Chinese hamster cell line CHL/IU (CA) and mouse lymphoma cell line L5178Y (Comet and mut)	(+)	(+) only after UV irradiation	NA	(+) only after UV irradiation	NA	(-) MLA	NA	Nakagawa 1997
TiO2 - Anatase	255	50-3200	1 h w/ or w/o UV		(+)	NA	NA	(+) w/ and w/o UV irr	NA	NA	NA	
TiO2 - Rutile	255	50-3200	1 h w/ or w/o UV		(-)	NA	NA	(-) w/ and w/o UV irr	NA	NA	NA	
TiO2 - Rutile	420	50-3200	1 h w/ or w/o UV		(-)	NA	NA	(+) only after UV irradiation	NA	NA	NA	
TiO2	20±7	0.5-5	24	Chinese hamster ovary cells	(+)	NA	(+)	NA	NA	NA	NA	Di Virgilio 2010
TiO2 - rutile	10 x 40	3.8-380	24-48-72	Human bronchial epithelial (BEAS-2B)	(+/-)	NA	(-)	(+/-)	NA	NA	NA	Falck 2009
TiO2 - anatase	<25				(+)	NA	(+)	(+)	NA	NA	NA	
TiO2 - rutile	<5 µm				(+)	NA	(-)	(+)	NA	NA	NA	
TiO2 - anatase	<25	10-40	60 days	Chinese hamster ovary cells	(-)	NA	NA	(-)	NA	(-) Hprt	NA	Wang 2011
TiO 2 - 70-85% anatase 30-15% rutile	<25	20-50-100	48 (MN); 6-12-24 (Comet)	Isolated human lymphocytes	(+)	NA	(+)	(+)	NA	NA	NA	Kang 2008
TiO2 P-25 (anatase:rutile 8:2)	<25	10-50-100 µg/cm2	24	human skin fibroblast (BJhTERT)	(-)	(-)	NA	NA	NA	NA	NA	Browning 2015
TiO2 anatase		26-65-130	6-24-48	human B-cell lymphoblastoid (WIL2-NS)	(+, 130 µg/mL)	NA	(+)	(+)	NA	(+) Hprt	NA	Wang 2007
TiO2 P-25 (anatase:rutile 8:2)	<25	0.01-0.1-1	24	human fetal hepatocyte (L-02)	(-)	NA	(-)	(-)	(+, 1 µg/mL) 8-OHdG (HPLC)	NA	NA	Shi 2010

NPs	Primary Size (nm)	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Chromosome aberrations	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Cell transformation	Reference
TiO2 anatase	75±15	5-20-100	2-6-24	Chinese hamster lung fibroblasts (V79 cells)	(+, 24 h)	NA	NA	(+, 100 µg/mL, 24 h)	NA	(+, 20-100 µg/mL, 2 h) Hprt	NA	Chen 2014
TiO2 anatase	11-18	2001-05-10	24-72	Balb/3T3 mouse fibroblasts	(-)	NA	(-)	NA	NA	NA	(-) CTA	Uboldi 2016
TiO2 rutile	10-35				(+, 72 h)		(+)				(+) CTA	
bulk TiO2 anatase	60-400				(-)		(-)				(-) CTA	
bulk TiO2 rutile	250-600				(+, 72 h)		(-)				(+) CTA	
TiO2 (anatase:rutile 8:2)	<100	1-5-10-20-40	2-24	human lung epithelial (A549)	(+, 40 µg/mL)	NA	NA	(+, 2 h) (-, 24 h)	(+, 2 h)	NA	NA	Ursini 2014
				Human bronchial epithelial (BEAS-2B)	(+, 10 to 40 µg/mL)			(-)	(-)			
TiO2 P-25 (anatase:rutile 8:2)	<25	1-2.5-5-10-50	24	human lung epithelial (A549)	(-)	NA	(-)	(+, 50 µg/mL)	(+, 10-50 µg/mL)	NA	NA	Armand 2016
			1 week					(+, 50 µg/mL)	(+, 10-50 µg/mL)			
			2 weeks					(+, 50 µg/mL)	(+, 1-50 µg/mL)			
			1 month					(+, 10-50 µg/mL)	(+, 5-50 µg/mL)			
			2 month					(+, 10-50 µg/mL)	(+, 1-50 µg/mL)			
TiO2 (anatase:rutile 8:2)	21	0.1-1-10-100	24	human fibroblast cells (GM07492)	(+, 100 µg/mL)	NA	NA	(-)	(-)	NA	NA	Franchi 2015
TiO2 P-25 (anatase:rutile 8:2)	21	0.12-0.6-3-15-75 µg/cm2	2-24	human lymphoblast cells (TK6)	(-)	NA	NA	(-)	(-)	NA	NA	Magdolenova 2012
					(-)			(-)	(+, 2 h, 75 µg/cm2)			
			2-24	monkey kidney fibroblasts (Cos-1)	(+)			(+, 75 µg/mL)	(+, 24 h, 3 µg/cm2)			
			24	human embryonic ephitelial cells (EUE)	(-)			(-)	NA			
					(-)			(+, 75 µg/cm2)	NA			

7.1.5 Table V. In vivo Au

Nanoparticle	Primary particle size (nm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
Au (citrate)	10 30 10 30	male Wistar rats	single intraperitoneal administration once daily for 28 days intraperitoneal administration	70 µg/Kg	24 h post-exp.	comet assay in blood and liver cells	Both the acute and chronic administration of 10 and 30 nm Au NPs induced DNA damage in blood and liver cells.	Cardoso 2014
Au (citrate)	10 30 10 30	male Wistar rats	single intraperitoneal administration once daily for 28 days intraperitoneal administration	70 µg/Kg	24 h post-exp.	comet assay in cortex cells	Both the acute and chronic administration of 10 and 30 nm Au NPs induced DNA damage in cortex cells. The chronic administration shows higher DNA damage respect to the acute treatments.	Cardoso 2014b
Au colloid suspensions in water	2 20 200	male Wistar rats	single intratracheal instillation	18 µg per lung	3 days post-exp.	bone marrow micronucleus assay and comet assay in the lung cells	No MN induction and weak size-related DNA damage increase with high animal-to-animal variation.	Schulz 2012
Au colloid suspensions in water	2 20 200	male Wistar rats	3 intravenous injection	6 µg Au/animal	4, 24, 48 hrs post-exp.	Comet assay (blood, liver and lung tissues), MN-reticulocytes assay by flow cytometer	no genotoxicity. Inductively coupled plasma-mass spectrometry data showed increased Au levels in liver and lung tissues.	Downs 2012
Au colloid suspensions in water	40 100	male, female and pregnant Swiss mice	single intraperitoneal administration	3.3 mg/kg	24 h post-exp.	bone marrow MN assay and fetal liver and blood MN assay	100 nm AuNPs increased the frequency of MN in both fetal livers and peripheral blood whereas no genotoxicity was detected in pregnant, male and female mice	Balansky 2013

7.1.6 Table VI. In vitro Au

NPs	Primary Size (nm)	Capping agent/surf.	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Chromosome aberrations	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Cell transformation	Notes	Reference
Au	5	citrate	0.1-100	48 h (MN), 2 and 24 h (comet)	Whole peripheral blood	(+)	NA	(++)	(++)	(++)	NA	NA	Aneuploidy was induced irrespective of the Au NPs size, and the lymphocytes showed a relatively higher percentage of chromosome mis-segregation than macrophages.	Di Bucchianico 2014
					murine macrophages (Raw264.7)			(+)	(++)	(+)				
	15				Whole peripheral blood			(+++)	(++)	(++)				
					murine macrophages (Raw264.7)			(+++)	(++)	(+)				
Au	20	citrate	0.1-100 µM	24 h	human liver cells (HepG2)	(-)	NA	NA	(+)	NA	NA	NA	No significant differences were observed in the rate of internalization between Citrate and MUA-AuNPs.	Fraga 2013
		11-mercaptoundecanoic acid							(-)					
Au	7.3 ± 1.2	citrate	1 µM	3 h	peripheral blood mononuclear cells	(+)	NA	NA	(-)	NA	NA	NA	A statistically significant ($p < 0.05$) measurement of intracellular ROS was observed for both HepG2 and PBMC upon treatment with AuNps	Paino 2012
	7.2 ± 2.7	PAMAM	1 µM		human liver cells (HepG2)				(+)					
	7.3 ± 1.2	citrate	50 µM		peripheral blood mononuclear cells				(+)					
	7.2 ± 2.7	PAMAM	50 µM		human liver cells (HepG2)				(++)					
Au	20	citrate	1 nM	72 h	human fetal lung fibroblast cells (MRC-5)	NA	(+)	NA	(+)	NA	NA	NA	As shown by FISH, all aberrations observed were chromosomal breaks with the majority being undetectable telomeres.	Li 2011
Au	2.3	graphitic carbon and enolate ions	0.0046-4.6	48 h	human lung epithelial (A549)	(++)	NA	(++)	NA	NA	NA	NA	Gold nanoparticles were obtained by laser ablation	Di Bucchianico 2015
	7.1	none				(-)		(-)						
	2.4	graphitic carbon				(+)		(+)						

7.1.7 Table VII. In vivo CNTs

Nanoparticle	Diameter (nm)	Length (µm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
N-MWCNTs	44	2.7	male CrI/CD(SD) rats	single intratracheal instillation repeated intratracheal inst. once a week for 5 weeks	0.2 mg/Kg 1 mg/Kg 0.2 mg/Kg 1 mg/Kg 0.04 mg/Kg 0.2 mg/Kg	3 h after inst. 24 h after inst. 3 h after inst.	comet assay in the lung cells	Histopathological examinations of the lungs revealed that all MWCNTs exposures caused inflammatory changes including the infiltration of macrophages and neutrophils without DNA damage.	Ema 2012
MWCNTs (CM-100)	10-15	20	male Fischer 344 rats female Fischer 344 rats	nose-only (6 h/day, 5 days/week, 28 days)	0.2-1 mg/m^3	0 days post-exp. 90 days post-exp. 0 days post-exp. 90 days post-exp.	comet assay in the lung cells	MWCNTs induced DNA damage in both male and female rats from all the exposed groups. The DNA damage was retained even 90 days post-exposure.	Kim 2014
Carboxylated-SWCNTs	1	10	male Swiss–Webster mice	intraperitoneal injection (once a day for 5 days)	0.25-0.75 mg/kg	24 h post-exp.	bone marrow micronucleus assay and chromosome aberration, comet assay (mice leukocytes)	Carboxylated-SWCNTs exposure significantly increased structural chromosomal aberrations and the frequency of micronuclei in bone marrow cells as well as induced DNA damage in mice leukocytes.	Patlolla 2015

Nanoparticle	Diameter (nm)	Length (µm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
MWCNTs Carboxylated-MWCNTs	15-30	15-20	male Swiss–Webster mice	intraperitoneal injection (once a day for 5 days)	0.25-0.75 mg/kg	24 h post-exp.	bone marrow micronucleus assay and chromosome aberration, comet assay (mice leukocytes)	Carboxylated-MWCNTs induced a higher genotoxicity compared to non-functionalized MWCNTs	Patlolla 2010
MWCNTs (Graphistrengt h© C100)	12	1	male and female Wistar rats	nose-only (6 h/day, 5 days/week, 90 days)	0.05, 0.25 and 5.0 mg/m^3	24 h post-exp.	bone marrow micronucleus assay and comet assay (lung, kidney and liver cells)	No genotoxicity was detected locally in lung and distally in bone marrow, liver and kidney.	Pothmann 2015
SWCNTs	65	1-3	female C57BL/6 mice	whole body inhalation (5 mg/m3, 5 h/day, for 4 days)	40 µg/mouse	1 yr post-exp.	Lung karyological assay	Genotoxicity of SWCNT was demonstrated by a significant increase in micronuclei and micronuclei plus protrusions in lung cells. Accumulation of K-ras mutations in lungs.	Shvedova 2014
MWCNTs (straight) MWCNTs (tangled)	70 15	4.5 0.4	female C57Bl/6 mice	Pharyngeal aspiration	1 to 200 µg/mouse	24 h post-exp.	Comet assay (BAL and lung cells), γ-H2AX assay (lung cells and blood leukocytes)	Straight MWCNTs induced a dose-dependent increase in DNA strand breaks in the lung cells	Catalán 2016

Nanoparticle	Diameter (nm)	Length (µm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
MWCNTs (straight)	70	4.5		Inhalation exposures	8.2± 1.7 mg/m^3	4 h per day during 4 days	Comet assay (BAL and lung cells), γ-H2AX assay (lung cells and blood leukocytes) and MN assay in bone marrow and lung cells	The level of DNA damage and MN frequency was significantly increased by straight MWCNTs	
MWCNTs (tangled)	15	0.4			17.5± 2.0 mg/m^3				

7.1.8 Table VIII. In vitro CNTs

NPs	Diameter (nm)	Length (µm)	Capping agent/surf.	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Chromosome aberrations	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Cell transformation	Reference
Nanocyl-7000	9.5	1.5	none	1-100	24 (MN), 72 (cell transformation)	mouse fibroblasts (Balb/3T3)	(-)	NA	(-)	NA	NA	NA	(+)	Ponti 2013
Nanocyl-3101			-COOH										(++)	
Nanocyl-3152	9.5	<1	-NH2										(+)	
Nanocyl-3153			-OH										(+)	
NRCWE-006	70	4.5	none	up to 100 µg/cm^2 (comet) and 20 µg/cm^2 (MN)	24 (comet), 48 (MN)	human bronchial epithelial cells (BEAS-2B)	(+)	NA	(-)	(+)	NA	NA	NA	Catalán 2016
NRCWE-007	15	0.4	none	up to 200 µg/cm^2 (comet) and 100 µg/cm^2 (MN)			(-)		(-)	(+)				
MWCNT (NM403)	12±7	0.4 ±0.2	none	1-20	24, 1 week, 3 weeks	human bronchial epithelial cells (BEAS-2B)	at least 70% viability	NA	(+)	(-)	(-)	NA	(+) soft agar	Vales 2016
MWCNT (NM401)	70	4	none	0.12-12 µg/cm^2	24	Chinese hamster lung fibroblasts (V79-4)	(+)	NA	NA	NA	NA	(+)	NA	Rubio 2016
SWCNT	400-800	NA	none	1-100	24-48	human bronchial epithelial cells (BEAS-2B)	(-)	NA	(++)	NA	NA	NA	NA	Manshian 2013
	1-3 µm								(+)			NA		
	5-30 µm								(+)					
	400-800								(++)			(-)		
	1-3 µm								(+)			(+)		
	5-30 µm		none	1-100	48	human lymphoblastoid B cells (MCL-5)	(-)		(+)			(-)		

NPs	Diameter (nm)	Length (µm)	Capping agent/surf.	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Chromosome aberrations	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Cell transformation	Reference
NM401	10-30	5-15	none	12.5-200	24	FE1-Muta(TM) Mouse lung epithelial cells (MML)	(-)	NA	NA	(-)	NA	NA	NA	Jackson 2015
NM402	NA	NA	none				(-)			(-)				
NM403	10-15	0.1-10	none				(-)			(-)				
NRCWE-006	40-90	NA	none				(-)			(-)				
NRCWE-040			none				(-)			(-)				
NRCWE-041	8-15	10-50	-OH				(-)			(-)				
NRCWE-042			-COOH				(-)			(+)				
NRCWE-043			none				(-)			(-)				
NRCWE-044	50-80	10-20	-OH				(-)			(*)				
NRCWE-045			-COOH				(-)			(-)				
NRCWE-046			none				(-)			(*)				
NRCWE-047	13-18	1-12	-OH				(-)			(*)				
NRCWE-048			-COOH				(-)			(-)				
NRCWE-049			-NH2				(-)			(*)				
MWCNT	10-30	0.5-50	none	1-10	48 (MN), 24 (Comet)	murine macrophages (Raw264.7)	(+)	(+)	(+)	(++)	NA	NA	NA	Di Giorgio 2011
SWCNT	1.2-1.5	2-5					(-)	(+)	(+)	(+)				
MWCNT	15-30	15-30	none	12.5	3 (MN), 1 (Comet)	human lung epithelial (A549)	(+)	NA	(+)	(++)	(++)	NA	NA	Visalli 2015
	15-30	0.2-1	-COOH				(-)		(-)	(+)	(+)			

*, significantly reduced tail length at the highest doses with concurrent reduced proliferation.

7.1.9 Table IX. In vivo Ag

Nanoparticle	Primary particle size (nm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
PVP-AgNPs	3-8	male B6C3F1 mice	intravenous injection	0.5, 1.0, 2.5, 5.0, 10.0 or 20.0 mg/kg	48 h post-exp (MN assay) and 48 h, 2 day or 2 and 6 weeks post-exp. (Pig-a assay)	MN assay (erythrocytes) and Pig-a assays (erythrocytes and/or reticulocytes)	no genotoxicity or mutagenicity	Li 2014
PVP-AgNPs	15-100			single 25 mg/kg or 25 mg/kg/day for 3 consecutive days	3 h post-exp.	comet assay and enzyme-modified comet assay (ENDOIII and hOGG1) in liver tissues	No DNA strand breaks were detected in liver while significant induction of oxidative DNA damage was found in the enzyme-modified comet assay	
silica-AgNPs	10-80							
AgNPs	60	male and female Sprague-Dawley rats	oral administration	30-300-1000 mg/Kg/day for 28 days	24 h post-exp.	MN assay (erythrocytes)	slight and not significant increase of MN frequency	Kim 2008
AgNPs	20	Ogg1-/- KO and WT C57BL/6 mice	intravenous injection	5 mg/kg	1 and 7-day post-exp.	comet assay and enzyme-modified comet assay (Fpg) in testis, lung and liver tissues	increased levels of DNA damage were observed 7-day post-exp. in lung tissues with Ag20 nm more effective than Ag200 nm in WT mice whereas Ag200 was more effective in inducing oxidative DNA damage both in lung and testis of WT exposed mice.	Asare 2015
sub-micro Ag	200							

Nanoparticle	Primary particle size (nm)	Model system	Treatment conditions	Dose	Time	Genotoxicity testing method	Results	Reference
AgNPs	75-130	Swiss albino male mice	Intraperitoneal Injection	10, 20, 40 and 80 mg/kg	18 h post-exp.	Bone marrow Chromosome aberration assay and comet assay	AgNPs induced chromatid breaks and DNA damage in bone marrow cells with concurrent ROS increase	Ghosh 2012

7.1.10 Table X. In vitro Ag

NPs	Primary Size (nm)	Capping agent/surf.	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Chromosome aberrations	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Cell transformation	Notes	Reference
Ag	<100	PVP	0.1-100	24	human fibroblast (GM07492)	(+, 100 µg/mL)	NA	NA	(+, 10 µg/mL)	(+, 10 µg/mL)	NA	NA	Ag NPs increased GADD45α transcript levels and the phosphorylation of proteins γH2AX	Franchi 2015
Ag	50	PVP	1.1-21.6 µg/cm2	2-24	human lung epithelial (A549) and Chinese hamster lung fibroblasts (V79-4) for Hprt mutation assay	(+)	NA	NA	(++)	(+)	(+)	NA	inflammatory cytokines (IL-8 and MCP-1) production induced by all NPs	Huk 2014
	80	PVP				(+)			(+)	(+)	(+)			
									(+)		(++)			
	200	PVP				(+)				(+)				
Ag	5.9±2.3	3-sodium citrate (-)	0.3-3 µg/cm2	2-24	human B-lymphoblastoid (TK6) and Chinese hamster lung fibroblasts (V79-4) for Hprt mutation assay	(-)	NA	NA	(+)	(+)	(+)*	NA	Severe genotoxic effects of cationic Ag ENMs can be combined with the presence of Ag ENMs in the nucleus and mitochondria, which suggests that Ag ENMs can induce toxicity by both direct contact with DNA and indirect (via oxidative stress) mechanisms. *, mutagenic effects of stabilizer	Huk 2015
	6.2±2.9	SDS (-)				(-)			(+, 24 h)	(+)	(+, 2.5 µg/cm2)			
	10.5±2.5	BYK-9076 (+)				(+, 2 and 24 h, 2.5 µg/cm2)			(++)	(++)	(++)			
	9.8±2.1	Chitosan in acetic acid (+)				(+, 2 and 24 h, 3 µg/cm2)			(++)	(++)	(+, 0.6 µg/cm2)			
	6.9±2.8	Disperbyk (neutral)				(-)			(+, 24 h)	(+)	(+, 2.5 µg/cm2)			
	6.1±2.1	Tween 80 (neutral)				(-)			(+, 24 h)	(+)	(+)*			
Ag	42.5±14.5	PVP	0.5–48 µg/cm2	4-24 (Comet)-48 (MN)	human bronchial epithelial (BEAS-2B)	(+)	(-)	(-)	(+)	NA	NA	NA	The extensive NPs agglomeration could interfere with the assays	Nymark 2013

NPs	Primary Size (nm)	Capping agent/surf.	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Chromosome aberrations	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Cell transformation	Notes	Reference
Ag	10	none	0.025-2.5	24 h	Chinese hamster ovary fibroblasts (CHO-K1)	(+, 5 µg/mL)	NA	(+, 2.5 µg/mL)	(+, 0.25 µg/mL)	NA	NA	NA	Both NPs in both cell lines modulate the cell cycle as assessed by flow cytometry	Souza 2016
	100							(-)	(+, 1.25 µg/mL)					
	10				Chinese hamster ovary fibroblasts (CHO-XRS5)			(-)	(+, 1.25 µg/mL)					
	100							(+, 2.5 µg/mL)	(+, 1.25 µg/mL)					
Ag	15.9±7.6	none	1-10	24 h	Chinese hamster ovary fibroblasts (CHO-K1)	(+, 5 µg/mL)	NA	(+)	(+)*	(++)**	NA	NA	*, bulky DNA adduct; **, 8-oxodG. Decreased mitochondrial activity, increased intracellular ROS and induced apoptosis were also shown	Jiang 2013
AgNO3	soluble							(++)	(++)*	(+)**				
Ag	NA	none	0.1-10	24 h	Chinese hamster ovary fibroblasts (CHO-K1)	(-)	NA	(+)*	(+)	NA	(-)**	NA	*, co-exposures with Cytochalasin-B affected MN induction; **, mutagenicity in *Salmonella typhimurium* test strains	Kim 2013
Ag	12-40	Polyvinyl alcohol	20-100	48 h	human fibroblasts (IMR-90)	(-)	NA	(-)	(-)***	NA	NA	NA	*, normal level of DNA-PKcs; **, deficient in DNA-PKcs expression and activity; ***, pre-treatment with NU7026 (DNA-PKcs inhibitor) significantly increased the NP-induced DNA damage. DNA repair deficiency or inhibition enhanced NP-induced genome instability	Lim 2012
					human glioblastoma (M059K)*	(+, 60 µg/mL)		(+)***	(+)***					
					human glioblastoma (M059J)**	(-)		(-)	(+)					
					Chinese hamster ovarian (CHO-AA8)*	(+, 80 µg/mL)		(-)***	(-)***					
					Chinese hamster ovarian (CHO-V33)**	(+, 40 µg/mL)		(+/-)	(+/-)					

NPs	Primary Size (nm)	Capping agent/surf.	Range of test concentrations (µg/mL)	Time exposure (h)	Cell type	Cytotoxicity	Chromosome aberrations	Micronuclei	DNA damage	Ox DNA damage	Mutagenicity	Cell transformation	Notes	Reference
Ag	<10	none	3.7-256	48 h	whole blood lymphocytes	(+)	(+)	(+)	NA	NA	NA	NA	AgNPs induced chromatid breaks in a concentration-dependent manner	Joksić 2016
Ag	4-12	none	4-6	4 h	mouse lymphoma (L5178Y)	(+)	NA	NA	(-)	(+)	(+) MLA	NA	Genes involved in production of ROS, oxidative stress response and DNA repair changed their expression	Mei 2012
Ag	<50	none	0.01-10	1-3-24 h	Human adipose-tissue derived mesenchymal stem cells	(+, 10 µg/mL)	(+, 0.1 µg/mL)	NA	(+, 0.1 µg/mL)	NA	NA	NA	Increase of IL-6, IL-8 and VEGF release; migration ability was not impaired at subtoxic concentrations	Hackenberg 2011
Ag	6-20	Starch	25-400 (Comet) and 100-200 (MN)	48 h	Human glioblastoma cells (U251)	(+)	NA	(+)	(+, 50 µg/mL)	NA	NA	NA	Mitochondrial dysfunction and ROS induction; cells arrested at G2/M interface	AshaRani 2009
					Normal human fibroblasts (IMR-90)	(+)	NA	(+)	(+, 25 µg/mL)	NA	NA	NA		
Ag	10	citrate	10	4-24	human bronchial epithelial (BEAS-2B)	(+)	NA	NA	(+)	NA	NA	NA	There were no signs of DNA damage at earlier time points (4 h) suggesting indirect genotoxic.	Gliga 2014
	40					(-)			(+)					
	75					(-)			(+)					
	10	PVP				(+)			(+)					
	50	none				(-)			(+)					

KEMI
Swedish Chemicals Agency

Box 2, SE-172 13 Sundbyberg
+46 8 519 41 100

Visitors' and delivery address
Esplanaden 3A, Sundbyberg

kemi@kemi.se
www.kemikalieinspektionen.se