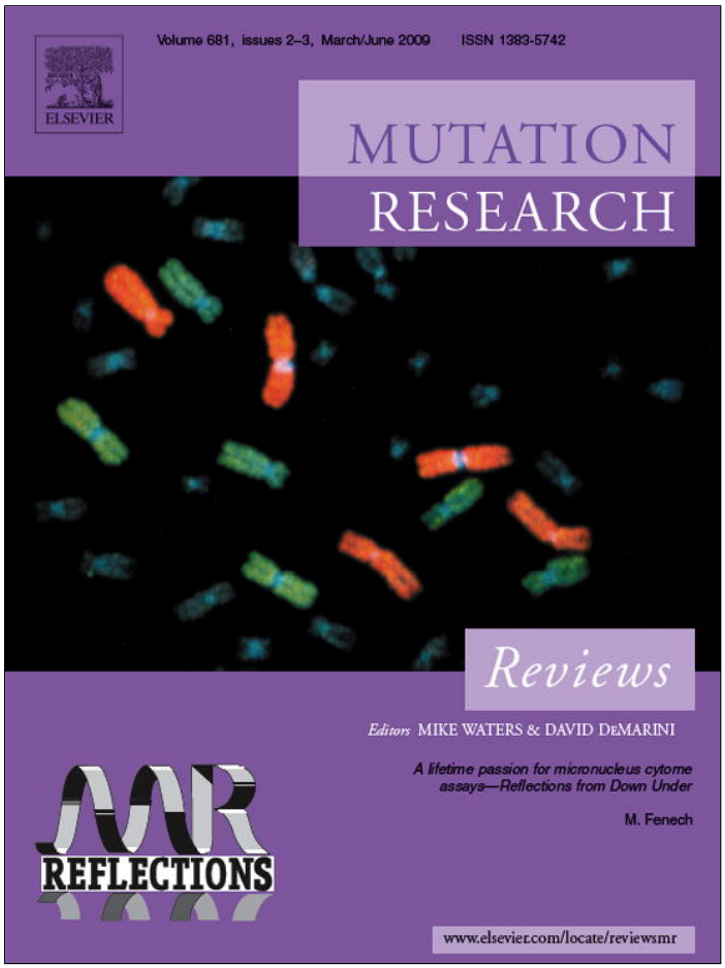

Mutation Research 681 (2009) 241–258

Contents lists available at ScienceDirect

Mutation Research/Reviews in Mutation Research

journal homepage: www.elsevier.com/locate/reviewsmr
Community address: www.elsevier.com/locate/mutres

Review

# Genotoxicity investigations on nanomaterials: Methods, preparation and characterization of test material, potential artifacts and limitations—Many questions, some answers

Robert Landsiedel [a], Maike Diana Kapp [b], Markus Schulz [a], Karin Wiench [a], Franz Oesch [c,*]

[a] Department of Product Safety, BASF SE, 67056 Ludwigshafen, Germany
[b] University of Applied Sciences, 68163 Mannheim, Germany
[c] Institute of Toxicology, University of Mainz, 55131 Mainz, Germany

ARTICLE INFO

*Article history:*
Received 2 September 2008
Received in revised form 28 October 2008
Accepted 29 October 2008
Available online 11 November 2008

*Keywords:*
Nanomaterials
Particles
Mutagenicity
Genotoxicity
DNA damage
Test methods

ABSTRACT

Nanomaterials display novel properties to which most toxicologists have not consciously been exposed before the advent of their practical use. The same properties, small size and particular shape, large surface area and surface activity, which make nanomaterials attractive in many applications, may contribute to their toxicological profile. This review describes what is known about genotoxicity investigations on nanomaterials published in the openly available scientific literature to-date. The most frequently used test was the Comet assay: 19 studies, 14 with positive outcome. The second most frequently used test was the micronucleus test: 14 studies, 12 of them with positive outcome. The Ames test, popular with other materials, was less frequently used (6 studies) and was almost always negative, the bacterial cell wall possibly being a barrier for many nanomaterials. Recommendations for improvements emerging from analyzing the reports summarized in this review are: Know what nanomaterial has been tested (and in what form); Consider uptake and distribution of the nanomaterial; Use standardized methods; Recognize that nanomaterials are not all the same; Use in vivo studies to correlate in vitro results; Take nanomaterials specific properties into account; Learn about the mechanism of nanomaterials genotoxic effects. It is concluded that experiences with other, non-nano, substances (molecules and larger particles) taught us that mechanisms of genotoxic effects can be diverse and their elucidation can be demanding, while there often is an immediate need to assess the genotoxic hazard. Thus a practical, pragmatic approach is the use of a battery of standard genotoxicity testing methods covering a wide range of mechanisms. Application of these standard methods to nanomaterials demands adaptations and the interpretation of results from the genotoxicity tests may need additional considerations. This review should help to improve standard genotoxicity testing as well as investigations on the underlying mechanism and the interpretation of genotoxicity data on nanomaterials.



**Contents**



* Corresponding author at: Rheinblick 21, D-55263 Wackernheim, Germany. Tel.: +49 6132 574 96/174 910 6 911; fax: +49 6132 577 59.
*E-mail address:* oesch@uni-mainz.de (F. Oesch).

1383-5742/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrrev.2008.10.002

## 1. Introduction

Nanomaterials are generally defined as having one or more external dimensions or an internal or surface structure on the nanoscale (about 1–100 nm). Nano-objects are nanomaterials with at least one external dimension and nanoparticles with all three external dimensions on the nanoscale (according to ISO/TC229). Nanomaterials display novel properties to which most toxicologists have not consciously been exposed before the advent of their practical use for many purposes ranging from applications in medicine to various industrial products from catalysts and electronics to paints and cosmetics [1,2 and references therein]. There are realistic prospects of substantial benefits to medicine, environment and energy efficiency. Yet, the very same properties, small size and particular shape, large surface area and surface activity, which make nanomaterials attractive in medicine and many other applications, may contribute to their toxicological profile.

Sensational titles in the internet such as "First nanotechnology genotoxicity tests find that carbon nanotubes could damage DNA" (by Michael Berger. Copyright 2007 Nanowerk LLC) may suggest that very little has been done with respect to investigate the genotoxicological safety of nanomaterials or at least of some important classes of them. Fortunately the reality to be outlined in this review is not as bad as this. The above-mentioned 2007 report details that researchers at the University of Dayton have assessed the DNA damage response to multi-walled carbon nanotubes (MWCNT) in mouse embryonic stem cells referring to work by Dr. Liming Dai and inferring to use p53 as a biomarker for preliminary screening of genotoxicity of nanomaterials on grounds of the close relationship between p53 activation and DNA damage. However, the majority of genotoxicity studies with nanomaterials available in the literature rather employed generally used genotoxicity tests with these novel materials.

This review describes what is known about genotoxicity investigations on nanomaterials published in the openly available scientific literature to-date. Reports on genotoxicity tests with positive or negative outcome will be summarized and genotoxicity tests which gave positive versus negative results depending on the particle size or on the test used will be contrasted. For convenience for the reader the preparation and characterization of the material and the test conditions, as far as specified in the literature, will be given at each of these places in the review and the sequence of the presented individual studies is methodologically oriented primarily on the genotoxicity test method used.

## 2. Genotoxicity tests reported to have been used on nanomaterials with positive results

### 2.1. Tests on DNA damage with positive outcome

According to the reports on genotoxicity tests on nanomaterials found in independent literature searches by two scientists to be summarized in this review the most frequently used genotoxicity test for nanomaterials was the single-cell gel electrophoresis assay (designated in the following by its more popular name Comet test): 19 studies, 14 with positive outcome, 5 with negative outcome (totally 26 studies on DNA damage, 20 positive, 6 negative). Those with positive outcome will be briefly presented here, those with negative outcome further below (in Section 3).

In 2006 Dhawan et al. [3] reported that aqueous suspensions of colloidal C60 *fullerenes* free of toxic organic solvents prepared by either ethanol to water solvent exchange ("EtOH/nC60 suspensions") or by mixing in water ("aqu/nC60 suspensions") evaluated using the Comet assay on human lymphocytes both were genotoxic with a strong correlation between the genotoxic response and nC60 concentration, and with genotoxicity observed at concentrations as low as 2.2 μg/L for aqu/nC60 and 4.2 μg/L for EtOH/nC60. The Olive tail moments (OTM, a quantitative measure of the genotoxic damage detectable by the Comet assay) for these two lowest concentrations were $1.54 \pm 0.24$ and $1.34 \pm 0.07$, respectively, which in comparison with the negative control OTM of $0.98 \pm 0.17$ was statistically different ($p < 0.05$). Aqu/nC60 suspensions elicited higher genotoxic response than EtOH/nC60 for the same nC60 concentration [3]. It should be noted, however, that the size of C60 is <1 nm, i.e. it is "sub-nano" and, although considered by many as a nanomaterial, it should be clearly separated from true nanomaterials, not only because its size is technically clearly below the nanometer range, but especially because its properties are very different from those of true nanomaterials.

*Single-walled carbon nanotubes (SWCNT)* are nanomaterials with extremely small diameters. Kisin et al. [4] investigated the genotoxicity of SWCNT with diameters ranging from 0.4 to 1.2 nm, a length of 1–3 μm and a surface area of 1040 $m^2/g$, comprised of 99.7 wt% element carbon and iron levels of approximately 2.3 mg Fe/g sample (0.23 wt%). SWCNT were

ultrasonicated (30 s × 3 cycles) and investigated by the Comet assay in V79 cells seeded into Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS) at concentrations of 0, 24, 48 or 96 μg/cm$^2$. Already after only 3 h of incubation with 96 μg/cm$^2$ of SWCNT the Comet assay showed significant DNA damage (4.2-fold increase of OTM in comparison with vehicle treated cells) [4].

Nanoparticles of *cobalt chrome alloy* caused in alkaline Comet assay in human fibroblasts (tested concentrations 0.0005–5000 μm$^3$) after 24 h of exposure DNA damage in a dose-dependent manner (tail moment [TM] at highest dose of 5000 μm$^3$/cell about 17-fold compared with control cells). The CoCr nanoparticles were generated using a flat pin-on-plate tribometer followed by sonication in sterile, pyrogen-free water for 1 h. The tested material had a mean size of 29.5 ± 6.3 nm and the shape was round to oval, determined after coating with 5–10 nm platinum/palladium by viewing in field emission gun electron microscopy. The composition of the particles was 62.2% Co, 28.7% Cr, 6.3% Mo, 0.87% Si, 0.71% Ni, 0.59% Mn, 0.53% Fe, 0.057% C, similar to the composition in orthopaedic joint replacement protheses. The primary human dermal fibroblasts used for testing were suspended in MEM containing 10% FCS and 2 mM L-glutamine. The test particles were sonicated for 30 s in the culture medium prior to test. It may be of technical interest that after 3 days of exposure the tested DNA damage was very much smaller (but was still significant) [5].

*Titanium dioxide ($TiO_2$)* (99% purity) particles suspended in culture medium (RPMI 1640 with 5% FCS) were sonicated, spun at 78 g for 5 min and the supernatant fraction was sterilized by filtration. The particle size distribution in the final extract was by volume 6.57 nm: 100%, and by intensity 8.2 nm: 80.4% and 196.5 nm: 19.4% as determined by high-performance particle sizer. At a concentration of 65 μg/mL the nanoparticles induced after an exposure of 24 h in cultured human B-cell lymphoblastoid WIL2-NS cells in the alkaline Comet assay significant genotoxicity (5× increase of OTM) [6].

A recent study (K.B. Fischer and H.F. Krug, personal communication and Poster at INIS, Hannover 2008) compared the solubility, potential to generate reactive oxygen species, activity in the Comet assay and potential to produce micronuclei of four related metal oxide materials: *$V_2O_3$ and $V_2O_5$*, each of them as nanomaterial and as bulk material. $V_2O_5$ were more water soluble than $V_2O_3$, nanomaterial more than bulk material. The potential to generate reactive oxygen species correlated with the relative water solubility, yet the genotoxicity did not: of the four materials the most water soluble, the nano $V_2O_5$ and the least water soluble, the bulk $V_2O_3$ were negative in the Comet assay (on A549 cells, concentrations: 1 and 2 μg/cm$^2$, time: 24, 36, 48 h) (and also negative in the micronucleus assay).

Nanoparticulate *Carbon Black* Printex 90 (primary diameter 14 nm, suspended at 100 μg/mL in serum-free Dulbecco's Modified Eagle's Medium (DMEM) and sonicated for 20 min) caused in treated A549 (a type II alveolar-like human lung adenocarcinoma cell line) after 3 h exposure a significant increase in single-strand (but not double strand) DNA breaks and alkali-labile sites as differentiated in the alkaline/neutral Comet assay [7].

Nanosized (10 and 20 nm) anatase *$TiO_2$* particles were sterilized (120 °C, 2 h) and suspended in sterilized phosphate-buffered saline (PBS) (10 μg/mL). The nanoparticles induced in human bronchial epithelial cells (BEAS-2B, cultured in LHC-9 medium containing 10% FCS and ~2 mM glutamate) in the alkaline Comet assay with and without formamidopyrimidine glycosylase (FPG) oxidative DNA damage determined as strand breaks and as base damage in terms of sites sensitive to FPG which cleaves DNA at sites of oxidized purines and mainly detects 8-oxodG. The Comet assay was performed in total darkness to insure absence of photocatalysis [8].

*Carbon Black particles* (Printex 90, primary particle size: 14 nm, specific surface area: 295 m$^2$/g) were sonicated in 5 mL medium using a Branson Sonifier S-450D equipped with a disruptor horn, with 10 s pulses alternating with 10 s pauses for a total sonication time of 4 min. Organic impurities were ~1%. Of the 16 EPA priority polycyclic aromatic hydrocarbons (PAH) phenanthrene, fluoranthene, pyrene, benzo[*a*]anthracene, and chrysene were detected. FE1 Muta$^{TM}$Mouse lung epithelial cells were cultured in DMEM F12 (1:1) medium, 2% FCS, 2 mM L-glutamine, 1 ng/mL murine epidermal growth factor. 75 μg/mL particles induced in these cells within 3 h a significant increase in DNA strand breaks ($p = 0.02$) and oxidized purines ($p = 0.008$) detected in the alkaline Comet assay with and without FPG [9].

*Diesel exhaust particles* (74% with an aerodynamic diameter below 0.13 μm determined by a cascade impactor and a differential mobility analyzer in connection with a condensation particle counter) suspended in complete culture medium (Ham's F12 with 10% FCS and 2 mM glutamine) and sonicated led in human lung epithelial A549 cells incubated with 100 or 500 μg/mL in the alkaline Comet assay to increases in tail length which were small (<1.5 fold) but at $p < 0.05$ significant after 2, 5 and 24 h of treatment [10].

In the same study female 8-week-old transgenic Muta$^{TM}$mice were exposed by inhalation to an aerosol of 20 or 80 mg/m$^3$ (6 or 73 × 10$^5$ particles/cm$^3$ as quantitated by a condensation particle counter) *diesel exhaust particles*. 74% of the particles had an aerodynamic diameter below 0.13 μm, determined as described just above in the preceding paragraph. Treatments were either a single exposure or the same dose fractionated into four exposures on four consecutive days in a nose-only inhalation chamber. These treatments led in lung tissue in the alkaline Comet assay to increases in tail length after repeated exposure to 4 × 90 min to 5 or 20 mg/m$^3$. In broncho-alveolar lavage cells these treatments led to increases in 8-oxoG (determined by electrochemical detection) after single exposures to 80 mg/m$^3$ and to bulky DNA adducts (determined by $^{32}$P postlabelling) after repeated exposure to 4 × 90 min to 5 or 20 mg/m$^3$ [10].

Nanoparticles (ultrafine particles of 10–100 nm) from *vehicle exhaust* (determined by particle counters carried with the inlet tube in the breathing zone during bicycling in traffic) led in mononuclear blood cells compared with bicycling indoors to a 4-fold increase in oxidative DNA base damage in terms of FPG-sensitive sites determined by alkaline Comet assay with FPG, but to no effect on DNA strand breaks as assayed in the absence of FPG. The blood cells had been suspended in preservation medium (40% RPMI, 50% FCS, 10% dimethyl sulfoxide) and stored at −80 °C [11].

A study by the Public Health group of the University of Copenhagen and their collaborators in Benin, Africa [12] showed in four locations of differently heavy traffic clear stepwise gradients of both, concentration of nanoparticles (0–320,000 particles per cm$^3$) pollution and degree of genotoxic damage in peripheral mononuclear blood cells of exposed people as detected by the alkaline Comet assay with FPG expressed as % DNA in the tail. However, the correlation was just as good with the gradient of benzene concentrations in these different locations (and further common genotoxic agents in traffic emissions such as PAH were not measured in the same study). The particle size range and number was determined by portable particle counters TSI 3007 to be 0–320,000 particles with 10–1000 nm in diameter per cm$^3$ during continuous measurement [12].

In a subsequent study by the Public Health group of the University of Copenhagen [13] persons were specifically exposed to *urban air particles of defined size ranges* (average diameters 12, 23, 57 and 212 nm) (NC 6169–15,362 cm$^{-3}$) or to filtered air (NC 91–542 cm$^{-3}$) in exposure chamber. The size distribution and

number concentration of particles were monitored using a differential mobility particle sizer. Exposure to nanoparticles (ultrafine particles) for 6 or 24 h significantly increased the levels of strand breaks and FPG sensitive sites in peripheral mononuclear blood cells detected by the alkaline Comet assay. Interestingly, not the smallest nanoparticles were most effective. The maximal damage was rather reached with particles of a medium diameter of 57 nm (the "57-nm soot fraction from vehicle emissions") with less damage by particles of smaller or larger size [13].

For exposure to UV–vis light from a sunlight simulator (50 min) L5178Y mouse lymphoma cells and *TiO$_2$* P25 particles, anatase form, average size 21 nm were suspended in Earl's Balanced Salt Solution (EBSS). After irradiation the cells were suspended in MEM culture medium with 10% FCS for the alkaline Comet assay which showed a dose-dependent increase in tail length which increased with increasing UV energy from 1.6 to 5 J/cm$^2$. No increase in tail length was seen without irradiation [14].

*TiO$_2$* samples (20–50 nm in diameter) were extracted from over-the-counter sunscreens by washing with organic solvents (methyl cyanide, acetone, chloroform), and their anatase and rutile contents were determined by X-ray diffraction. Anatase and rutile standards were a gift. Some samples also contained ZnO (no precise composition of the different sunscreen samples was given). Samples with a concentration of 0.0125% (w/v) $TiO_2$ were illuminated in the presence of MRC-5 human fibroblasts by a solar simulator giving an intensity similar to that found upon natural sunlight exposure under the stratum corneum. This combined treatment, but not $TiO_2$ without illumination, led to oxidative damage to DNA as determined by the alkaline Comet assay. - Illumination of supercoiled plasmids with simulated sunlight and 0.025% (w/v) $TiO_2$ mixed with an equal volume of DNA showed in agarose gel electrophoresis that plasmids were converted to the relaxed form and to the linear form, demonstrating strand breakage. Sunlight alone had very little effect, 100% anatase standard was more active than 100% rutile standard, and $TiO_2$ extracted from a sunscreen was also photo-active, and so was pure ZnO. The sunscreen extractions contained much less $TiO_2$ than the anatase and rutile standards, suggesting that the sunscreen variety is especially active [15].

Commercially available water-soluble semiconductor *CdSe/ZnS quantum dots* (cadmium selenide capped with a shell of zinc sulphide, complete with biotin surface functionality) were incubated with supercoiled double strands of plasmid DNA which was then precipitated and run on an electrophoresis gel. The exposure to the quantum dots led to DNA damage whether the incubation was under UV light (56% damaged DNA) or in the dark (29% damaged DNA) suggesting that the damaging mechanism for DNA was not a simple photo-induced free radical process. Plasmid damage was observed in assays run with DNA isolated from dots at time 0, indicating immediate modification upon mixing the dots and the DNA. It may be of technical interest that the general intensity of all bands in assays with DNA incubated with quantum dots were much weaker than in experiments without quantum dots. The authors attributed this to coordination of dots with DNA during incubation, resulting in smaller yields of isolated DNA estimating up to 70% of the DNA coordinated to the dots and therefore unavailable for assay analysis [16]. This may have consequences for limits of sensitivity.

*Gold nanoparticles* were prepared by $NaBH_4$ reduction of $KAuCl_4$ and characterized by Transmission Electron Microscopy (TEM) analyzing the size distribution by ImageJ as 1–12 nm with 87% below 5 nm. The gold nanoparticles were prepared and mixed with DNA within 12 h to maintain the particle size distribution. For longer time periods aggregation can be reduced by ligand exchange method. DNA complexes formed by mixing pGEM-3Zf (−) plasmid DNA with gold nanoparticles irradiated by 60 keV electrons showed sensitisation to irradiation-induced DNA damage by the gold nanoparticles, This was visualized by neutral agarose gel electrophoresis. The formation of single strand and double strand breaks of DNA from exposure to irradiation by fast electrons was increased by 1:1 and 2:1 gold nanoparticle:DNA ratios about 2.5-fold compared with pure DNA suggesting that targeting the DNA of cancer cells with gold nanoparticles may increase the effectiveness of radiotherapy [17].

*Nanoparticulate nickel powder* (mean diameter 20 nm, surface area 43.8 m$^2$/g), metallic cobalt powder (mean diameter 20 nm, surface area 47.9 m$^2$/g; composed of Co and $Co_3O_4$) and nanoparticle $TiO_2$ (mean diameter 28 nm, surface area: 45 m$^2$/g) (size distributions determined by TEM) were dispersed in distilled water and sonicated for 30 s. For investigation of potential damage to DNA 8 μL of buffer (pH 7.2) containing 290 ng φX 174 RF1 DNA were added to 10 μL of water containing 1, 10, or 20 μg of nanoparticles and incubated for 8 h at 37 °C. Agarose gel electrophoresis showed depletion of supercoiled DNA by 20 μg of either Co nanoparticles or Ni nanoparticles of 70–75%, by 20 μg of $TiO_2$ of about 15% [18].

Size-selected aerosol *wildfire smoke samples* were collected with the Micro-Orifice Uniform Deposit Impactor (MOUDI), a cascade impactor including a final filter to collect particles <56 nm. The mass concentration of particulates ranged from 0.75 to 1.3 mg/m$^3$. Filter suspensions were prepared by splitting the filters into size groups defined as ultrafine (42–240 nm), fine (0.42–2.4 μm) and coarse (4.2–24 μm) particles. The 42–240 nm particle fraction (100 μg/mL) was allowed to react for 30 min at 37 °C with 10 μg DNA (lambda Hind III fragments) in the presence of 1 mM $H_2O_2$ (to partially simulate wildfire smoke conditions). This caused DNA damage apparent in the form of smeared bands (caused by DNA being cut into randomly sized pieces) in DNA strand break neutral agarose gel assay [19].

*SWCNT* were produced employing CO as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor and purified by acid treatment to remove metal contaminates. Analysis by NMAN 5040 and ICP-AES showed that SWCNT comprises 99.7% (wt) elemental carbon and 0.23% (wt) iron. TGA-DSC, TPO, NIR, and Raman spectroscopy demonstrated that >99% of carbon content in the SWCNT product had a carbon nanotube morphology. The mean diameter and surface area were 1–4 nm and 1.04 m$^2$/g, respectively. Surface area was determined by Brunauer, Emmett, and Teller (BET) analysis, and diameter was measured by TEM. C57BL/6 mice were exposed (by pipetting onto the base of the tongue) to 10 or 40 μg/mouse SWCNT (prepared by sonification in PBS for 3 min at room temperature). Aortic mitochondrial DNA damage (significant reduction in mitochondrial DNA amplification visualized by quantitative polymerase chain reaction) was observed at 7, 28, and 60 days after exposure. Also repeated exposure to SWCNT (20 μg/mouse once every other week for 8 weeks) led to increased mitochondrial DNA damage [20].

Chen et al. [21] report a "simple new electrochemical method to detect DNA damage caused by photovoltaic effect of *nano-titanium dioxide*": Substrate electrode: Gold. DNA and nano-$TiO_2$ were co-modified onto the surface of the gold electrode to prepare the working electrode. Reference electrode: Saturated calomel. Counter electrode: Platinum wire. Final concentration 0.1 mg/mL $TiO_2$ and 0.1 mg/mL DNA. Irradiation under UV light for 90 min. The authors conclude that the method visualizes that ROS produced from $TiO_2$ nanoparticles can oxidatively damage DNA [21].

#### 2.1.1. Brief overview on Section 2.1

Tests on DNA damage have very successfully been performed with nanomaterials. 20 tests on DNA damage with nanomaterials

were positive (6 negative, see Section 3.1). The most frequently used test was the Comet assay: 19 studies, 14 with positive outcome, the latter on the following materials (all in vitro except where stated): fullerenes [3], SWCNT [4], cobalt chrome alloy [5], $TiO_2$ [6,8,14,15], $V_2O_3$ and $V_2O_5$ (K.B. Fischer and H.F. Krug, personal communication and Poster at INIS, Hannover 2008), Carbon Black [7,9], Diesel exhaust particles (in vitro and ex vivo) [10], general traffic vehicle exhaust (ex vivo) [11], urban and rural air pollution (ex vivo) [12], urban air particles of defined size ranges (ex vivo) [13]. In 4 tests on DNA damage with positive outcome supercoiled double strands of plasmid DNA were analyzed by agarose gel electrophoresis after exposure to the following nanomaterials: CdSe/ZnS quantum dots [16], Gold nanoparticles [17], nickel powder [18], wildfire smoke samples [19]. One study observed damage to mitochondrial DNA visualized by quantitative polymerase chain reaction upon in vivo exposure of mice to SWCNT [20] and one study reported on photovoltaic DNA damage by $TiO_2$ investigated by electrochemical method [21] (Table 1).

### 2.2. Tests on Gene mutations with positive outcome

Only 11 genotoxicity studies on nanomaterials which used gene mutation tests were identified in the publically available scientific literature, 6 of them with a positive outcome as detailed below.

Water-soluble *nano-FePt* were prepared by mixing $Fe(OEt)_3$, $Pt(acac)_2$, oleic acid and oleamine. Nano-FePt was separated by centrifugation and characterized by TEM, X-ray diffraction, energy X-ray, FT-IR, CHNS elemental analysis and SQUID which showed a mean diameter of 9 nm and a face-centered cubic crystal structure. From this material FePt nanoparticles capped with tetramethylammonium hydroxide were prepared by ligand exchange with tetramethylammonium hydroxide against oleic acid. This material (78.1–5000 μg/plate) tested in the Ames Salmonella strains TA 98, TA 100, TA 1535 and TA 1537 and *E. coli* strain WPA2*uvr*A/pKM101 with and without exogenous metabolic activation by S9 mix in the preincubation method (8 h preincubation) was weakly positive in the TA100 strain without S9 mix (maximal specific mutagenicity 61.6 revertants/mg) [22].

*$SiO_2$* nanoparticles (99% purity, particle size distribution measured using HPPS: by volume 7.21 nm: 100%; by intensity 9.08 nm: 71.4% and 123.21 nm: 28.6%) were suspended in culture medium (RPMI 1640 supplemented with 5% FCS), vortexed for 10 min and sonicated for 10 min in an ultrasonic water bath. In WIL2-NS human B-cell lymphoblastoid cells exposed to 120 μg/mL $SiO_2$ nanoparticles for 24 h the mutation frequency was significantly ($p < 0.05$) increased by $3.8 \times 10^{-5}$ (above a background of $6.8 \times 10^{-5}$) as detected by the hypoxanthine guanine phosphoribosyltransferase (HPRT) mutation assay [23,24].

*$TiO_2$* nanoparticles (99% purity, particle size distribution measured using HPPS: by volume 6.57 nm, 100%; by intensity 8.2 nm, 80.4% and 196 nm, 19.4%) were suspended in culture medium (RPMI 1640 supplemented with 5% FCS) and spun at 78 g for 5 min and the supernatant sterilized by filtration. In WIL2-NS human B-cell lymphoblastoid cells exposure to $TiO_2$ nanoparticles for 24 h led to a mutation frequency of $15 \times 10^{-6}$ using 130 μg $TiO_2$ nanoparticles per mL and to $10 \times 10^{-6}$ using 65 μg/mL compared with $6 \times 10^{-6}$ for the untreated control ($p < 0.05$) as detected by the HPRT mutation assay [6].

Similar to the principle of the frequently used HPRT mutation assay (used in the experiments above) the adenine phosphoribosyltransferase (Aprt) can be used to detect mutagenicity since Aprt-deficient cells can be selected in the presence of adenine analogues, such as 2-fluoroadenine, which are metabolized by Aprt to cytotoxic products. Aprt-heterozygous mouse (C3H/Hej) embryonic stem cells (maintained in DMEM supplemented with 15% embryonic stem cell-quality FCS, 0.1 μM β-mercaptoethanol and 50 μM recombinant leukemia inhibitory factor) were treated with *Multi-Walled Carbon Nanotubes (MWCNT)* (size unspezified) for 4 h. 5 μg/mL increased the mutation frequency by ~2 fold compared with the untreated embryonic stem cells (from $4.2 \times 10^{-5}$ to $7.8 \times 10^{-5}$) [25].

Sub-cytotoxic concentrations (75 μg/mL) of *Carbon Black* Printex 90 were weakly genotoxic at the *lacZ* and *cII* transgene loci of the FE1 Muta™Mouse lung epithelial cell line following eight repeated 72-h incubations, each with 75 μg/mL (cumulative dose $8 \times 75$ μg $\times$ 10 mL = 6 mg) Carbon Black (mutant frequency 1.4 fold [95% CI: 1.22–1.58] for *cII* and 1.23 fold [95% CI: 1.10–1.37] for *lacZ* compared with identically passaged untreated cells). The Carbon Black particles had a primary particle size of 14 nm and a specific surface area of 295 $m^2$/g and were sonicated in 5 mL medium using a Branson Sonifier S-450D equipped with a disruptor horn, with 10 s pulses alternating with 10 s pauses for a total sonication time of 4 min. Organic impurities were ~1%. Five (phenanthrene, fluoranthene, pyrene, benzo[*a*]anthracene, and chrysene) of the 16 EPA priority PAH were detected in an extract of Carbon Black. The detected PAHs are only weakly mutagenic compared with benzo[*a*]pyrene, and were present in very low amounts (sum of these five PAH 74.2 ng/g). The authors rightfully conclude that it is highly unlikely that the increase in mutant frequency following Carbon Black exposure stems from the PAH content. FE1 Muta™Mouse lung epithelial cells were cultured in DMEM F12 (1:1) medium, 2% FCS, 2 mM L-glutamine, 1 ng/mL murine epidermal growth factor [7]. It may be technically interesting that the mutant frequency for the *cII* gene of the negative control cells increased 1.27 fold (statistically significant at $p = 0.007$; 95% CI: 1.08–1.45 fold) following chloroform/phenol extraction. The authors suggest that the increase might be caused by DNA damage induced during the chloroform/phenol extraction since it had been demonstrated that phenol-based extraction can introduce oxidative DNA damage [26].

*HPRT* mutations were increased in type II alveolar lung cells isolated from rats which had been treated by tracheal instillation with 100 mg/kg nanoparticulate *Carbon Black* (but not significantly after treatment with 10 mg/kg). The animals used were specific pathogen free female F344 Fischer rats. The particles had a diameter of 15 nm and a surface area of 230 $m^2$/g, were sterilized at 200 °C for 2 h and instilled suspended in 2 mL/kg saline. 15 months after instillation *hprt* mutations in type II alveolar lung cells were increased about 7 fold (estimated from the figure). In vitro addition of bronchoalveolar lavage cells from the rats 15 months after their treatment with 100 mg/kg nanoparticulate Carbon Black (but not from saline-treated control rats) led to a significant ($p < 0.05$) about 4-fold increase in *hprt* mutation of RLE-6TN cells (a rat lung epithelial cell line) suggesting a significant contribution of inflammatory cell products to the genotoxicity of the Carbon Black nanoparticles (the increase in mutation frequency was observed at a ratio 50:1 bronchoalveolar lavage cells: RLE, but not at a ratio 10:1) [27]. It may be worth to remember that the clearance of particles from the lung of rats is less efficient compared with that from other species [28].

#### 2.2.1. Brief overview on Section 2.2

Bacterial gene mutation assays on nanomaterials were seldom positive (only one Ames test was positive, only weakly positive and in a single strain; investigated material: Water-Soluble FePt nanoparticles capped with $(CH_3)_4NH_4OH$) [22] (while 5 Ames tests were negative, see Section 3.2). Perhaps penetration of the test material through the bacterial cell wall is a problem. Five mammalian gene mutation assays were positive (while 2 were

**Table 1**
Genotoxicity tests with nanomaterials.[a]

| Reference | Material | Characteristics/Preparation | Test systems | Results |
|---|---|---|---|---|
| Auffan et al. [37] | DMSA-coated Maghemite Nanoparticles | Roughly spherical, mean diameter 6 nm, surface 172 $m^2$/g, coated with DMSA yielding nanoparticles with a negative surface charge to prevent aggregation | Fibroblasts from infant foreskin analyzed in the alkaline comet assay | Comet negative, attributed in part to the DMSA coating, which serves as a barrier between nano-maghemite and fibroblasts, inhibiting a potential toxic effect |
| Avogbe et al. [12] | Particles from air of different locations | 0–320,000 particles with 10–1000 nm in diameter per $cm^3$ air during continuous measurement | Alkaline Comet Assay ± FPG in mononuclear blood cells of exposed humans | Air with high levels of benzene and nanoparticles was positive in the Comet Assay with FPG |
| Bräuner et al. [13] | Urban air particles | Average diameters 12, 23, 57 and 212 nm | Participants were exposed to urban air particles in exposure chamber. Mononuclear blood cell DNA was investigated by Alkaline Comet Assay ± FPG | Increase of SBs and FPG-sensitive sites. The 57 nm fraction caused the highest yield of DNA damage |
| Chen et al. [21] | Nano-titanium dioxide | Not described | After irradiation by UV detection of DNA damage by electrochemical method:<br>Substrate electrode: DNA and nano-$TiO_2$ co-modified onto gold electrode<br>Reference electrode: Saturated Calomel<br>Counter electrode: Platinum wire | DNA damage caused by photovoltaic effect of nano-titanium dioxide |
| Driscoll et al. [27] | Carbon Black | Particles (diameter 15 nm, surface 230 $m^2$/g) were suspended in saline | Tracheal instillation into rats followed by isolation of type II alveolar lung cells and HPRT mutation assay | HPRT mutation positive, a significant contribution by inflammation being suggested |
| Dufour et al. [31] | Zinc oxide (ZnO) | Micronised uncoated ZnO (mean diameter 100 nm, >99% pure) formulated as a 10% emulsion for Ames Test and CHO cells | • (Photo) Ames test with TA98, 100, 1573 and *E. coli* WP2<br>• Chromosome aberration in CHO cells | • Non-mutagenic in Ames test<br>• Clastogenic in vitro |
| Dunford et al. [15] | Titanium dioxide and zink oxide from sunscreens | $TiO_2$ samples (20–50 nm in diameter) with different anatase/rutile ratios were extracted from sunscreens (some samples also contained ZnO; precise composition of the different samples not given) | Agarose Gel Electrophoresis of supercoiled plasmid and Alkaline Comet Assay in human MRC-5 fibroblasts ± irradiation from solar simulator | Positive in Agarose Gel Electrophoresis of supercoiled plasmid and in Comet Assay after combined treatment with sunscreen extract + irradiation |
| Dybdahl et al. [10] | Diesel exhaust particles | 74% of the particles had an aerodynamic diameter below 130 nm. For in vitro exposure particles were sonicated in complete Ham's F12. For in vivo exposure an aerosol of 20 or 80 mg/$m^3$ was used | • Alkaline Comet Assay in human lung epithelial A549 cells and, after exposure by inhalation, in lung tissue of Muta™Mouse<br>• Determination of 8-oxo-deoxyguanosine in broncho-alveolar lavage cells of Muta™Mouse after exposure by inhalation<br>• Determination of *cII* mutation frequency in lung tissue of Muta™Mice after exposure by inhalation | • Comet positive<br>• Increase in 8-oxo-deoxyguanosine<br>• No increase in the *cII* mutation frequency in lung tissue of Muta™Mice |
| Freitas et al. [33] | Magnetite nanoparticles | Obtained by co-precipitation of Fe (II) and Fe (III) ions in alkaline medium, then pre-coated with dodecanoic acid followed by ethoxylated polyalcohol to obtain a stable sample, average core particle diameter 9.2 nm | Particles were intraperitoneally applied to mice and micronuclei scored in polychromatic erythrocytes | Increase in micronuclei in polychromatic erythrocytes |
| Green et al. [16] | CdSe/ZnS quantum dots | Commercially available semiconductor CdSe/ZnS quantum dots | Quantum dots were incubated with supercoiled DNA, precipitated DNA was analyzed by gel electrophoresis | DNA damage visualized by gel electrophoresis |
| Gurr et al. [8] | Titanium dioxide | $TiO_2$ anastase particles (10 and 20 nm) were suspended in phosphate-buffered saline | • Alkaline Comet assay ± FPG in BEAS-2B human bronchial epithelial cells<br>• Cytokinesis Block Micronucleus assay<br>Both assays performed in darkness | • Comet positive<br>• Micronuclei formation |

**Table 1** (*Continued*)

| Reference | Material | Characteristics/Preparation | Test systems | Results |
|---|---|---|---|---|
| Jacobsen et al. [9] | Carbon Black | Particles (primary particle size 14 nm, surface area 295 $m^2$/g) were sonicated in DMEM F12 | Alkaline Comet assay ± FPG and mutant frequency in FE1 Muta™Mouse lung epithelial cell line | DNA strand breaks and FPG sensitive sites<br>Weak increase in the mutant frequency in *cII* and *lacZ* |
| Kim et al. [41] | Silica-overcoated magnetic nanoparticles labeled with rhodamine B isothiocyanate (MNPs@$SiO_2$(RITC)) | Size: 50 nm | • Bacterial reverse mutation test with S. typhimuriumTA97, TA98, TA100, TA102, all with and without S9 mix | • Ames negative |
| | | | • Chromosome aberration assay in Chinese hamster lung fibroblasts | • No increase in chromosome aberrations |
| Kisin et al. [4] | Single-walled Carbon Nanotubes (SWCNT) | SWCNT (99.7%t carbon, 0.23% iron, diameter 0.4–1.2 nm, length 1–3 μm, surface 1040 $m^2$/g) were ultrasonicated | • Comet assay in V79 | • Comet positive |
| | | | • Ames Salmonella Assay with strains YG1024 and YG1029 without S9 mix | • No increase in mutation frequencies in YG1024 or YG1029 |
| | | | • Micronucleus Assay | • Limited but not statistically significant micronucleus induction |
| Leonard et al. [19] | Wildfire smoke samples | 42–240 nm particle fraction collected by a cascade impactor | Particles were mixed with DNA in the presence of 1 mM $H_2O_2$. DNA was analyzed by neutral agarose gel assay | DNA was cut into randomly sized pieces |
| Li et al. [20] | Single-walled carbon nanotubes (SWCNT) | 99.7% carbon, 0.23% iron, >99% of carbon had a carbon nanotube morphology, mean diameter 1–4 nm, surface 1.040 $m^2$/g. Suspensions were prepared by sonification in PBS | Mice were exposed (by pipetting onto the base of the tongue) to SWCNT. Mitochondrial DNA damage was analyzed by quantitative polymerase chain reaction | Mitochondrial DNA damage was observed as a reduction in mitochondrial DNA amplification visualized by quantitative polymerase chain reaction |
| Linnainmaa et al. [42] | $TiO_2$ P25 and UV-TITAN M160 | • $TiO_2$ P25: Average crystal size 20 nm, uncoated anatase<br>• UV-TITAN M160: Average crystal size 20 nm, rutile coated by aluminium hydroxide and stearic acid, before exposure washed with ethanol to remove the stearic acid to make the particles hydrophobic and suspensable<br>• Pigmentary $TiO_2$ (170 nm, uncoated anatase)<br>All suspensions ultrasonicated | Cytokinesis Block Micronucleus Assay in rat liver epithelial cells. After 1 h of incubation, half of the slides were irradiated with an UV lamp | None of the $TiO_2$ samples increased the number of micronucleated cells. No synergism with the combined treatments by the test samples and UV |
| Maenoso et al. [22] | Water-Soluble FePt nanoparticles capped with $(CH_3)_4NH_4OH$ | Mean diameter 9 nm, face-centered cubic crystal structure | Ames Salmonella Assay with TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2*uvr*A/pKM101, with and without metabolic activation by S9 mix | Weakly positive in TA100 without S9 |
| Mroz et al. [7] | Carbon black | Particles (14 nm) were sonicated in DMEM | Alkaline/Neutral Comet Assay in A549 human adenocarcinoma cells | Comet positive |
| Muller et al. [32] | Multi-walled carbon nanotubes (MWCNT) | Particles (average outer diameter 11.3 nm, length 700 nm, in solutions/media aggregates with a hydrodynamic diameter of ~1 μm; 98% carbon, traces of cobalt and iron catalysts) were suspended in 0.9% saline containing 1% of Tween 80 for in vivo exposure, in Ham's F-12 for RLE cells, in RPMI 1640 for MCF-7 cells | • *Ex vivo* micronucleus test: The suspensions were intra-tracheally instilled in rats; type II pneumocytes were isolated after 3 days | • *Ex vivo* increase in micronucleated pneumocytes |
| | | | • *In vitro* Cytokinesis-block micronucleus assay in MCF-7 and RLE cells | • *In Vitro* increase of centromere-positive and -negative micronuclei |

**Table 1** (*Continued*)

| Reference | Material | Characteristics/Preparation | Test systems | Results |
|---|---|---|---|---|
| Nakagawa et al. [14] | Titanium dioxide P25 | For irradiation particles (Anatase form, average size 21 nm) were suspended in EBSS, for test in MEM, except for Salmonella/Microsome test in distilled water | • Alkaline Comet Assay in L5178Y mouse lymphoma cells | • Comet positive after irradiation |
| | | | • Chromosomal aberration assay in Chinese hamster CHL/IU cells | • Comet negative without irradiation |
| | | | • Salmonella/Micosome Assay with TA100, TA98 and TA102 | • Negative in the Ames Salmonella/Micosome assay with and without irradiation |
| | | | • Mammalian cell mutation assay in L5178Y mouse lymphoma cells. All tests: After 1 h incubation in the dark, the cells were exposed to UV–vis light for 50 min | • Negative in the mammalian cell mutation assay with and without irradiation<br>• Chromosomal aberrations increased after irradiation |
| Papageorgiou et al. [5] | Nanoparticles of CoCr alloy | Particles ($29.5 \pm 6.3$ nm, round to oval, 62.2% Co, 28.7% Cr, 6.3% Mo, 0.87% Si, 0.71 Ni, 0.59% Mn, 0.53% Fe, 0.057% C, similar to joint replacement protheses) were sonicated in MEM | • Alkaline Comet Assay in human fibroblasts | • Comet positive |
| | | | • Micronucleus assay in human fibroblasts, 12 h exposure to nanoparticle followed by 12 h cytochalasin B | • Increase in centromere-positive and -negative micronuclei |
| Park et al. [35] | Diffusion flame system as particle generator doped with iron or without iron | Estimated mean particle size 100 nm. The main hydrocarbons of the non-iron and iron-doped flame were toluene, butane, styrene, benzene and xylene | Mice were exposed in whole body inhalation chambers followed by isolation of splenic lymphocytes for chromosomal aberration assay and reticulocytes for micronucleus assay | • Increase of chromosome aberrations in the splenic lymphocytes |
| | | | | • Increase of micronuclei in reticulocytes |
| Rahman et al. [29] | Nanoparticulate Titanium Dioxide | Particles (<20 nm) were sterilized and suspended in phosphate-buffered saline | • Micronucleus assay in Syrian Hamster Embryo Fibroblasts | • Increase in micronuclei, no significant increase in kinetochore-positive micronuclei |
| | | | • Agarose gel electrophoresis | • The agarose gel electrophoresis revealed typical apoptotic structures |
| Rehn et al. [39] | $TiO_2$ P25, $TiO_2$ T805 | $TiO_2$ P25 (surface hydrophilic) and $TiO_2$ T805 (surface made hydrophobic by treatment with trimethoxyoctylsilane) were suspended in physiological saline supplemented with 0.25% lecithin. Primary particle diameter 20 nm, but particles were highly aggregated, sonication not leading to primary particles | Intratracheal instillation in rats followed by bronchoalveolar lavage for immunological determination of 8-oxoguanine | No increase of 8-oxoguanine |
| Sadeghiani et al. [34] | Magnetic nanoparticles | Obtained by co-precipitation of Fe(II) and Fe(III) ions in alkaline medium and then surface-coated with polyaspartic acid to obtain stability, average core particle diameter: 8.5 nm | Particles were intravenously injected in mice, micronuclei were scored in polychromatic erythrocytes | Micronuclei were increased in polychromatic erythrocytes |
| Theogaraj et al. [44] | Titanium dioxide | Tested materials: A, B, C: crystal anatase 80%, rutile 20%, primary particle size 21 nm; A: coating trimethoxy caprylylsilane, B: no coating, doped with 2% di-iron trioxide, C: no coating; D: 100% rutile, primary particle size 14 nm, coating 8–11% alumina and 1–3% simethicone; E: 100% anatase, aggregate size 60 nm, coating 37% alumina and 12–18% silica; F–H: 100% rutile, F: primary particle size 20 nm, coating 5–6.5% alumina and 1–4% dimethicone, G: primary particle size 15 nm, coating 3–8% alumina and 5–11% stearic acid; H: primary particle size 20–22 nm, coating 10.5–12.5% alumina and 3.5–5% silica. 30–150 nm aggregates are expected in the samples tested. For the chromosome aberration test A was dissolved in absolute ethanol, B and C in physiological saline, D–H in DMSO | Chromosome aberration test in CHO-WBL cells ± UV irradiation | None of the eight different forms of $TiO_2$ induced increases of chromosome aberrations either with or without UV irradiation |

**Table 1** (*Continued*)

| Reference | Material | Characteristics/Preparation | Test systems | Results |
|---|---|---|---|---|
| Vinzents et al. [11] | Ultrafine particles from vehicle exhaust | Particles of 10–100 nm | Alkaline Comet Assay ± FPG on mononuclear blood cells of exposed humans | Increase of FPG-sensitive sites, but no effect on DNA strand breaks in absence of FPG |
| Wang et al. [23,24] | $SiO_2$ nanoparticles | Particles (99% pure, size by volume 7.21 nm: 100%; by intensity 9.08 nm: 71.4% and 123.21 nm: 28.6%) were suspended in RPMI 1640, vortexed and sonicated | • HPRT Mutation Assay | • Induction of HPRT mutants |
| | | | • Cytokinesis Block Micronucleus Assay | • Increase in micronucleated binuclear cells |
| | | | • Comet Assay<br>All in WIL2-NS human B-cell lymphoblastoid cell line | • Comet negative |
| Wang et al. [6] | $TiO_2$ nanoparticles | Particles (99% pure, size by volume 6.57 nm: 100%, by intensity 8.2 nm: 80.4% and 196.5 nm: 19.4%) were sonicated in RPMI 1640 | • Alkaline Comet Assay | • Comet positive |
| | | | • HPRT Mutation Assay | • HPRT mutation positive |
| | | | • Cytokinesis Block Micronucleus Assay<br>All in cultured WIL2-NS human lymphoblastoid cells | • Increase in micronucleated binuclear cells |
| Wang et al. [30] | Nano-cerium-element-doped titanium dioxide (CDT) | CDT was prepared by dropping cerium sulphate solution into nano-anatase $TiO_2$ (13 nm) gel, then suspended in physiologic saline, sterilized and diluted with RPMI 1640 | • Micronucleus assay | Exposure to CDT for 4 h followed by irradiation with a 15 W fluorescent lamp led to an increase in micronuclei |
| | | | • Agarose gel electrophoresis<br>Both in Bel 7402 human hepatoma cell line | |
| Warheit et al. [40] | Ultrafine $TiO_2$ | Particles (79% rutile; 21% anatase, median size 140 nm, surface 38.5 $m^2/g$; ~90% $TiO_2$, 7% alumina, 1% amorphous silica) were suspended in water | • Bacterial reverse mutation test with S. typhimuriumTA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A (all with and without S9 mix) | • Ames negative |
| | | | • Chromosomal aberration in CHO cells ± Aroclor-induced rat liver S9 | • No increase in chromosome aberrations |
| Zhang et al. [18] | Nanoparticulate nickel powder, cobalt powder and titanium dioxide | *Nickel powder* mean diameter 20 nm, surface 43.8 $m^2/g$ | φX 174 RF1 DNA was mixed with nanoparticles. DNA damage was analyzed by agarose gel electrophoresis | Supercoiled DNA was depleted by $TiO_2 \ll Co \sim Ni$ |
| | | *Cobalt powder* mean diameter 20 nm, surface 47.9 $m^2/g$, composed of Co and $Co_3O_4$<br>*$TiO_2$* mean diameter: 28 nm, surface 45 $m^2/g$<br>Particles were dispersed in distilled water and sonicated | | |
| Zheng et al. [17] | Gold nanoparticles | Size 1–12 nm, 87% below 5 nm | Plasmid DNA mixed with gold nanoparticles was irradiated with 60 keV electrons, DNA damage was visualized by neutral agarose gel electrophoresis | Single and double strand breaks of DNA from irradiation by fast electrons were increased by gold nanoparticles |
| Zhong et al. [38] | Carbon Black | Particles (37 nm, 99% carbon) were sonicated in MEM | Alkaline Comet assay in V79 Chinese hamster lung fibroblasts and in Hel 299 human embryonic lung fibroblasts | Comet negative |
| Zhu et al. [25] | Multi-Walled Carbon Nanotubes | | Aprt mutation assay in Aprt-heterozygous mouse embryonic stem cells | Aprt mutation positive |

[a] Materials of a size <1 nm ("sub-nano" materials) such as fullerenes are not included in the table since their properties are very different from those of true nanomaterials.

negative, see Section 3.2). The positive mammalian gene mutation assays were on the following materials: $SiO_2$ [23,24], $TiO_2$ [6], MWCNT [25], Carbon Black [26,27], all studies in vitro except the last study which was ex vivo and in vitro (Table 1).

### 2.3. Tests on chromosome mutations with positive outcome

After the Comet assay (described under Section 2.1) one of the tests on chromosome mutations, namely the micronucleus assay, was the second most frequently used genotoxicity test on nanomaterials. Micronuclei are indicative of either numerical or structural chromosomal aberrations, and are known to be induced by a variety of genotoxic carcinogens.

Nanosized (10 nm) anatase *$TiO_2$* particles were sterilized (120°, 2 h) and suspended in sterilized PBS. At a concentration of 10 μg/mL the particles induced in human bronchial epithelial BEAS-2B cells in complete darkness to preclude photocatalysis within 24 h ~2.5-fold increased micronuclei formation as determined by the micronucleus assay with cytochalasin B to inhibit cytokinesis (the cells were cultured in LHC-9 medium containing 10% FCS and ~2 mM glutamate) [8].

*$SiO_2$* nanoparticles (99% purity, particle size distribution measured using HPPS: by volume 7.21 nm, 100%; by intensity 9.08 nm, 71.4% and 123.21 nm, 28.6%) were suspended in culture medium (RPMI 1640 supplemented with 5% FCS), vortexed for 10 min and sonicated for 10 min in an ultrasonic water bath. In WIL2-NS human B-cell lymphoblastoid cells exposed there was (as determined by the micronucleus assay with cytochalasin B) a significant dose-dependent increase in micronucleated binuclear cells with increasing dose of particles (a 4-fold increase compared with untreated cells at 120 μg/mL with the 24-h treatment; the lowest dose that gave a statistically significant increase was 30 μg/mL with 24-h treatment) [23,24].

*$TiO_2$* nanoparticles (99% purity, particle size distribution measured using HPPS: by volume 6.57 nm, 100%; by intensity 8.2 nm, 80.4% and 196 nm, 19.4%) were suspended in culture medium (RPMI 1640 supplemented with 5% FCS) and spun at 78 g for 5 min and the supernatant sterilized by filtration. In WIL2-NS human B-cell lymphoblastoid cells exposure to 130 μg $TiO_2$ nanoparticles per mL for 6 h led to an increase of ~2.5 fold in micronucleated binuclear cells as determined by the micronucleus assay with cytochalasin B [6].

Nanoparticulate *$TiO_2$* (<20 nm, sterilized at 120 °C for 2 h and suspended at 1 μg/μL in PBS) led in Syrian hamster embryo fibroblasts (cultured in modified Dulbecco's Eagle's reinforced medium supplemented with 15% FCS and 1% glucose) to a significant ($p \leq 0.05$) increase in micronuclei (24.5 to 31.3 micronuclei/1000 cells from 12 to 72 h of exposure; controls ~18 to ~23 micronuclei/1000 cells estimated from the figure). Kinetochore-positive micronuclei (visualized by CREST-staining) were only insignificantly increased indicating clastogenic but no aneugenic activity [29].

*CoCr* nanoparticles were generated using a flat pin-on-plate tribometer followed by sonication in sterile, pyrogen-free water for 1 h. The tested material had a mean size of 29.5 ± 6.3 nm and the shape was round to oval, determined after coating with 5–10 nm platinum/palladium by viewing in field emission gun electron microscopy. The composition of the particles was 62.2% Co, 28.7% Cr, 6.3% Mo, 0.87% Si, 0.71 Ni, 0.59% Mn, 0.53% Fe, 0.057% C, similar to the composition in orthopaedic joint replacement protheses. The particles were suspended in MEM and sonicated for 30 s. They caused in human fibroblasts (cultured in MEM with 10% FCS and 2 mM glutamine) exposed for 12 h a significant ($p < 0.001$) and dose-dependent (from 5 to 500 $\mu m^3$/cell) increase (up to ~2.5 fold estimated from the figure) in micronuclei as determined by the micronucleus assay with cytochalasin B (12 h instead of the standard 24 h) to inhibit cytokinesis. Centromere-positive and negative micronuclei were produced providing evidence for clastogenicity and aneugenicity. It may be of technical interest that the particles accumulated in the cytoplasm of the cells and at higher doses they tended to obscure the micronuclei (but did not obscure the fluorescent comets of the fragmented nuclei) [5].

*Nano-cerium-element-doped titanium dioxide (CDT)* was prepared by impregnation by dropping cerium sulphate aqueous solution into nano-anatase $TiO_2$ (13 nm) gel, then filtered, suspended in physiologic saline, sterilized and diluted with RPMI 1640 medium. Bel 7402 human hepatoma cells (cultured with RPMI 1640 containing 10% FCS and 1% 0.2 M L-glutamine) were exposed to 10 μg/mL CDT for 4 h and then irradiated for 10 min with a 15 W fluorescent lamp from a distance of 30 cm. This induced micronuclei (no further details on method or results concerning the micronucleus test were given) [30].

Uncoated *zinc oxide* nanoparticles (mean diameter 100 nm, >99% pure) were formulated as a 10% emulsion for CHO cells which were cultured in McCoy's 5A medium containing 10% FCS. About 1.5 h before harvest cells were treated with 1 μg/mL colchicine for arrest in metaphase. Chromosome aberrations (mostly chromatide deletions and exchanges) became statistically significant at 105 μg/mL (maximal incidence16%). UV irradiation increased the clastogenicity up to 45%, but when pre-irradiated and simultaneously irradiated cells were compared there was almost the same clastogenicity at equitoxic doses of nano zinc oxide (leading the authors to suggest that the observed modest increase by UV light does not constitute a true photo-clastogenicity) [31].

Chinese hamster CHL/IU cells (suspended in MEM with 10% FCS) were exposed to *$TiO_2$* P25 particles (anatase form, average size 21 nm) for 1 h in the dark. Then the medium was changed to EBSS and the cells were exposed to UV–vis light (5 J/$cm^2$ from a sunlight simulator) for 50 min. After treatment with demecolcin for arrest in metaphase 12.5 to 50 μg/mL $TiO_2$ led to a dose-dependent increase in chromosome aberrations (mostly chromatid breaks and exchanges). No increase in chromosome aberrations was seen without irradiation [14].

*MWCNT* (15 carbon layers on average) were synthesized by the decomposition of ethylene on alumina doped with a cobalt-iron catalyst mixture and purified by treatment with NaOH. Characterization of MWCNT by TEM showed an average outer diameter of 11.3 nm and a length of 700 nm. In the solutions/media used for exposure MWCNT aggregates were formed with a hydrodynamic diameter of ~1 μm. The carbon content determined by a STA-409 PC analyzer was 98%. The Co content determination by proton-induced X-ray emission with a proton beam of 2 MeV from a Tandetron accelerator showed traces of cobalt and iron catalysts. For in vitro cell exposure RLE epithelial cells were suspended in Ham's F-12 medium containing 5% FCS and 1% glutamine, MCF-7 cells in RPMI medium 1640 with 10% FCS. For in vivo exposure of female Wistar rats (200–250 g) MWCNT were sterilized (200 °C/2 h), suspended in a sterile 0.9% saline solution containing 1% Tween 80 and intra-tracheally instilled. In vitro there was a significant increase of micronuclei (up to 2 fold at the cytotoxic dose of 50 μg/mL) in RLE epithelial cells as determined by the micronucleus assay with cytochalasin B to inhibit cytokinesis. In MCF-7 cells centromere-positive and -negative micronuclei were produced providing evidence that MWCNT are clastogenic and aneugenic. In the ex vivo micronucleus test in type II pneumocytes isolated 3 days after administration of 0.5 or 2 mg to the rats there was a significant and dose-dependent maximally ~2-fold increase in micronucleated pneumocytes after a single administration [32].

$5 \times 10^{15}$, $5 \times 10^{16}$ and $5 \times 10^{17}$ particles/kg of *magnetite nanoparticles* (obtained by co-precipitation of Fe(II) and Fe(III)

ions in alkaline medium, then pre-coated with dodecanoic acid followed by ethoxylated polyalcohol to obtain a stable sample, average core particle diameter 9.2 nm) were intraperitoneally applied to Swiss mice. At the 2 higher concentrations a significant increase in micronuclei in polychromatic erythrocytes was observed 24 h after application (3.8–5 fold) [33].

Of a water-based magnetic fluid containing *magnetic nanoparticles* (average core particle diameter 8.5 nm as determined by TEM; obtained by co-precipitation of Fe(II) and Fe(III) ions in alkaline medium and then surface-coated with polyaspartic acid to obtain stability under physiological conditions) 50 μL containing about $0.6 \times 10^{16}$ or $1.6 \times 10^{16}$ particles/mL were intravenously injected in Swiss mice. Micronuclei were increased in polychromatic erythrocytes on the first and seventh day after treatment with $1.6 \times 10^{16}$ particles/mL and on the first day only after treatment with $0.6 \times 10^{16}$ particles/mL. After 30 days the micronucleus frequency was the same as in control animals [34].

Park et al. [35] used a *diffusion flame system as particle generator doped with iron or without iron*. They estimated the mean particle size to be 0.1 μm although their particle counter (Met one A237H) could not screen for particles <0.1 μm or >1 μm, but "because the percentage for the 0.1 and 0.2 μm sizes occupied 86–94%". The main hydrocarbons of the non-iron and iron-doped flame were toluene, butane, styrene, benzene and xylene. 5–6 weeks old B6C3F1 mice were exposed for 6 h per day, 5 days per week in whole body inhalation chambers. Using the iron-doped flame 2 weeks of exposure led to a significant increase in chromosome aberrations in the splenic lymphocytes isolated from the exposed mice at the highest particle concentration (400 $\mu g/m^3$), 4 weeks of exposure to an increase in chromosome aberrations at all concentrations investigated (100, 200 and 400 $\mu g/m^3$). Using the non-iron-doped flame increases in chromosome aberrations were only seen after 4 weeks at the highest concentration. In contrast, the non-iron-doped flame (and the iron-doped flame) led to an increase of micronuclei (visualized in the "supravital micronucleus assay") in the reticulocytes collected from the blood of mice already after 2 weeks exposure at the intermediate and highest concentrations [35].

#### 2.3.1. Brief overview on Section 2.3

Many (15) tests on chromosome mutations with nanomaterials were positive, especially micronucleus assays (12) (while 3 tests on chromosome aberration were positive) (negative: 2 micronucleus assays and 3 chromosome aberration tests, see Section 3.3). The positive micronucleus assays described in Section 2.3 (all in vitro except were stated) were performed with the following materials: $TiO_2$ [6,8,29], $SiO_2$ [23,24], CoCr [5], nano-cerium-element-doped $TiO_2$ ("CDT") [30], zinc oxide [31], $TiO_2$ + UV–vis irradiation [14], MWCNT in vitro and ex vivo [32], magnetite ex vivo [33,34], diffusion flame system as particle generator doped with iron or without iron ex vivo, the main hydrocarbons of the non-iron and iron-doped flame being toluene, butane, styrene, benzene and xylene [35]. Three chromosome aberration studies were positive, all of them in vitro. They were performed on the following materials: zinc oxide [31], $TiO_2$ (increase of chromosome aberrations only after UV–vis irradiation) [14] and diffusion flame system as particle generator doped with iron or without iron ex vivo, the main hydrocarbons of the non-iron and iron-doped flame being toluene, butane, styrene, benzene and xylene [35] (Table 1).

### 2.4. DNA damage dependent signalling/biomarkers/special methods

A few publications have appeared which measured genotoxic or potentially genotoxic effects by methods not in routine use for genotoxicity screening.

The report in the internet by Michael Berger (Copyright 2007 Nanowerk LLC) mentioned in the beginning of this review suggests to use p53 as a biomarker for preliminary screening of genotoxicity of nanomaterials on grounds of the close relationship between p53 activation and DNA damage.

A published paper by Mroz et al. [7] reports that nanoparticulate *Carbon Black* Printex 90 (primary diameter 14 nm, suspended at 100 μg/mL in serum-free DMEM and sonicated for 20 min) caused in treated A549 type II alveolar-like human lung adenocarcinoma cell line after 1 h increased p53 phosphorylation at serine 15 (9.6 fold) and phosphorylated p53BP1 (6.8 fold), after 3 h single-strand DNA breaks (Comet assay), and after 6 h phosphorylated BRCA1. N-acetylcysteine blocked the p-ser15-p53 response. Carbon black particles of larger size (260 nm) did not provoke any of these responses.

A publication by Hidaka et al. [36] reports on damage to DNA and RNA visualized by scanning micrographs at 2500× magnification after placing 5 mg of calf thymus DNA in a dispersion containing 20 mg *$TiO_2$* (commercial P25 $TiO_2$: 80% anatase, 20% rutile; BET surface area approximately 55 $m^2/g$) and 15 mL $H_2O$ and irradiating it for 0, 1 and 3 h in a Pyrex reactor at wavelengths longer than 290 nm with a 100 W Hg lamp.

## 3. Genotoxicity tests reported to have been used on nanomaterials with negative results

### 3.1. Tests on DNA damage with negative outcome

*Maghemite* nanoparticles (nano-$\gamma Fe_2O_3$) were synthesized via aqueous coprecipitation of $Fe^{2+}$ and $Fe^{3+}$ followed by oxidation yielding nanoparticles with a mean diameter of 6 nm (determined by TEM and X-ray diffraction) and a specific surface area of 172 $m^2/g$ (established by BET method). Nano-$\gamma Fe_2O_3$ were coated with DMSA, an organic molecule composed of two carboxylate and two thiolated functions yielding nanoparticles with a negative surface charge (NmDMSA) barrier to prevent aggregation. Fibroblasts from infant foreskin cultured in DMEM with 10% FCS, 2 mM L-glutamine and 1 mM sodium pyruvate were exposed to filter-sterilized NmDMSA. TEM showed the internalization of NmDMSA within fibroblasts (after 2 h of incubation). No evidence of aggregates in the cytoplasm or inside mitochondria or nucleus was found by TEM. In the alkaline Comet assay at concentrations from $10^{-6}$ to $10^{-1}$ g/L no significant increase of OTM was detected (while methylmethanesulfonate used as positive control yielded increases). This is attributed in part to the DMSA coating, which serves as a barrier for a contact between nano-$\gamma Fe_2O_3$ and fibroblasts, inhibiting a potential toxic effect [37].

*$TiO_2$* P25 particles, anatase form, average size 21 nm, suspended in MEM with 10% FCS, not exposed to UV–vis light, showed in L5178Y mouse lymphoma cells in the alkaline Comet assay at concentrations 3.1, 12.5, 50, 200, 800 μg/mL no increase in tail length [14].

*Carbon Black* of 37 nm particle size (99% carbon) autoclaved, sonicated in MEM, vortexed and sonicated again for 30 min did not increase the tail length in the alkaline Comet assay in V79 Chinese hamster lung fibroblasts (maintained in MEM, 10% FCS, 2 mM glutamine) and in Hel 299 human embryonic lung fibroblasts (maintained in MEM, 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 0.1% lactalbumin hydrolysate) exposed for 3 h to concentrations of 17.2–137.9 $\mu g/cm^2$ [38].

*$SiO_2$* nanoparticles (99% purity, particle size distribution measured using HPPS: by volume 7.21 nm, 100%; by intensity 9.08 nm, 71.4% and 123.21 nm, 28.6%) were suspended in culture medium (RPMI 1640 supplemented with % FCS), vortexed for 10 min and sonicated for 10 min in an ultrasonic water bath. In

WIL2-NS human B-cell lymphoblastoid cells exposed to 30, 60 or 120 μg/mL $SiO_2$ nanoparticles for 6, 24 or 48 h the alkaline Comet assay was negative (no increase in % tail DNA, tail length or OTM while all three parameters were increased after treatment with 0.1 mM $H_2O_2$ as a positive control) [23,24].

Nanoparticles (10–100 nm) from *vehicle exhaust* (determined by particle counters carried with the inlet tube in the breathing zone during bicycling in traffic) led in mononuclear blood cells compared with bicycling indoors to no increase in DNA strand breaks as determined by alkaline Comet assay (but to oxidative DNA base damage in terms of FPG-sensitive sites). The blood cells had been suspended in preservation medium (40% RPMI, 50% FCS, 10% dimethyl sulfoxide) and stored at −80° [10].

The microstructure and aggregation of *$TiO_2$* P25 (primary particle diameter 20 nm, particle surface hydrophilic) and $TiO_2$ T805 (primary particle diameter 20 nm, particle surface made hydrophobic by treatment with trimethoxyoctylsilane) were monitored by TEM. The titanium particles were slurried in an isopropanol/water mixture, agitated in an ultrasonic bath and transferred onto a TEM grid, dried and transferred into the column. TEM demonstrated that both types of $TiO_2$ were highly aggregated and suspension and intensive sonication in lecithin-supplemented PBS did not lead to primary particles of 20 nm in size. For intratracheal instillation of 0.15, 0.3, 0.6, and 1.2 mg/0.5 mL of $TiO_2$ P25 or $TiO_2$ T805 into adult female Wistar rats the particles were suspended in sterile physiological saline supplemented with 0.25% lecithin and sonicated for 5 min. At day 90 after instillation 8-oxoguanin was quantified by the rabbit anti-8-oxoGua polyclonal antibody, goat anti-rabbit-IgG $F(ab)_2$ fragments conjugated to rhodamine isothiocyanate followed by fluorescence image analysis in bronchoalveolar lavage cells. No increase of 8-oxoguanine over control was observed while there was a significant elevation of the of 8-oxoGua level in cells from rats instilled with quartz DQ12 as a positive control [39].

### 3.1.1. Brief overview on Section 3.1

Tests on DNA damage were frequently performed with nanomaterials (26 tests), of which only relatively few (6) were negative. Of the most frequently used test, the Comet assay, only 5 were negative (14 were positive, see Section 2.1). The Comet assays with negative outcome were performed on the following materials (all in vitro except where stated): Maghemite (nano-$\gamma Fe_2O_3$) coated with DMSA resulting in a negative surface charge ("NmDMSA") barrier to prevent aggregation and possibly responsible for the negative outcome of the Comet assay [37]; $TiO_2$ [14], Carbon Black [38], $SiO_2$ [23,24], vehicle exhaust (ex vivo) (no increase in DNA strand breaks as determined by Comet assay, but oxidative DNA damage in terms of FPG-sensitive sites) [11]. One negative test on DNA damage was ex vivo determination of 8-oxoguanine after intratracheal instillation of $TiO_2$ into rats [39] (Table 1).

## 3.2. Tests on gene mutations with negative outcome

Warheit et al. [40] used a well-characterized ultrafine $TiO_2$ consisting of 79% rutile and 21% anatase. X-ray fluorescence determined a composition of ~90 wt% $TiO_2$, 7% alumina, and 1% amorphous silica. Using dynamic light scattering, the median particle size was 140 nm in water (aqueous solution buffered in 0.1% tetrasodium pyrophosphate). The BET surface area was 38.5 $m^2/g$. The particle samples underwent neutralization of acidic chloride groups on the particle surface. The bacterial reverse mutation (Ames) test performed used the plate incorporation method and the *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2*uvr*A in the absence and presence of Aroclor-induced rat liver S9 as an exogenous metabolic activation system. The $TiO_2$ particles dissolved in sterile water (forming a homogeneous suspension up to the highest concentration tested) were negative at all dose levels which were 100, 333, 1000, 3333, and 5000 μg per plate [40].

Micronised uncoated *zinc oxide* (particle size <200 nm) formulated as a 10% emulsion were negative in the Ames test (strains TA98, TA100, TA1573 and *E. coli* WP2) (no further details provided) [31].

*SWCNT* with diameters from 0.4 to 1.2 nm, a length of 1–3 μm and a surface area of 1040 $m^2/g$, comprised of 99.7 wt% element carbon and iron levels of approximately 2.3 mg Fe/g (0.23 wt%) were ultrasonicated (30 s × 3 cycles). At concentrations of 60, 120, or 240 μg/plate they were negative in the Ames Salmonella assay using the strains YG1024 and YG1029 without S9 mix [4].

$TiO_2$ P25 particles (anatase form, average size 21 nm) were negative in the Ames Salmonella/Micosome assay at concentrations of 5000, 10,000, 20,000 and 40,000 μg/mL using the strains TA100, TA98 and TA102 with and without UV–vis light irradiation by a sunlight simulator (10 min, 1 $J/cm^2$ or 50 min, 5 $J/cm^2$). They were also negative in the mammalian cell mutation assay at concentrations of 250, 500, 750, 1000, 1250, 1500 and 2000 μg/mL with or without UV–vis irradiation for 50 min at 5 $J/cm^2$ using L5178Y mouse lymphoma cells (maintained in RPMI 1640, 1% sodium pyruvate, 0.05% pluronic F-68 and 10% horse serum and for the mouse lymphoma assay suspended in Earl's Balanced Salt Solution) [14].

Trimethoxysilane modified by rhodamine B isothiocyanate was mixed with tetraethoxysilane, injected into polyvinylpyrolidone-stabilized cobalt ferrite ethanol solution and polymerized on the surface of the cobalt ferrites to form 50 nm *silica-overcoated magnetic nanoparticles labeled with rhodamine B isothiocyanate: MNPs@$SiO_2$(RITC)*. The silica shell thickness was adjusted such that it could be reproduced by controlling the concentration of tetraethoxysilane. All MNPs@$SiO_2$ (RITC) were confirmed by TEM. 0.25, 0.5, and 1 mg MNP@$SiO_2$(RITC)/plate were tested in the bacterial reverse mutation direct plate incorporation Ames assay using strains TA97, TA98, TA100, and TA102. The concentrations of MNP@$SiO_2$(RITC) tested with and without S9 were 0.25, 0.5, and 1.0 mg per plate. When the number of reverted colonies was more than double the number of the negative control and was dose dependent, the result was considered as positive. The MNP@$SiO_2$(RITC) increased in a few instances the number of revertants in the Salmonella mutation assay; however, the pattern was neither reproducible nor did it reach the above criteria for being considered positive [41].

Female 8-week-old transgenic Muta™Mice were exposed by inhalation to an aerosol of 20 or 80 $mg/m^3$ (6 or 73 × $10^5$ particles/$cm^3$ as quantitated by a condensation particle counter) *diesel exhaust particles*. 74% of the particles had an aerodynamic diameter below 0.13 μm, determined by a cascade impactor and a differential mobility analyzer in connection with a condensation particle counter. Treatments were by single 90-min exposures or four 90 min treatments on four consecutive days using correspondingly fractionated doses in a nose-only inhalation chamber. No increase in the *cII* mutation frequency in lung tissue could be demonstrated 28 days after the last exposure [10].

### 3.2.1. Brief overview on Section 3.2

Gene mutation tests in bacteria (Ames test) were predominantly negative (5/6), possibly due to penetration problems of the nanomaterials through the bacterial cell wall. The following materials were tested in the negative Ames assays: $TiO_2$ [14,40], zinc oxide [31], SWCNT [4] (only 2 strains were used: YG1024 and YG1029 without S9 mix), silica-overcoated magnetic nanoparticles labeled with rhodamine B isothiocyanate "MNPs@$SiO_2$(RITC)" [41].

Two mammalian gene mutation tests were negative, one in vitro on $TiO_2$ [14], one ex vivo (*cII* mutation frequency in lung tissue of transgenic Muta™Mice exposed by inhalation to diesel exhaust particles) [10] (while 5 mammalian gene mutation assays were positive, see Section 2.2) (Table 1).

### 3.3. Tests on chromosome mutations with negative outcome

*$TiO_2$* P25 (uncoated anatase) and UV-TITAN MI60 (rutile coated by aluminium hydroxide and stearic acid, but before exposure washed with ethanol to remove the stearic acid in order to make the particles hydrophobic and suspensable), both with an average crystal size of 20 nm, and pigmentary $TiO_2$ (170 nm, uncoated anatase) were treated with ultrasound to make the suspensions more homogeneous. Rat liver epithelial cells were cultured in McCoy's 5A medium supplemented with 20% FCS and 0.03% L-glutamine. Semiconfluent cultures were treated with 0, 5, 10 and 20 pg/cm$^2$ P25, UV-TITAN MI60 or pigmentary $TiO_2$. After 1 h half of the samples were irradiated with UV (366 nm, 5 min). All cultures were treated with cytochalasin B (1 μg/mL) to block cytokinesis and further incubated for 20 h (37 °C). Binucleated cells with one, two, three, and more than three micronuclei were recorded. None of the $TiO_2$ samples increased the number of micronucleated cells while the positive control (1 μM mitomycin C) gave a strong induction of micronuclei. Exposure of the cells to UV alone gave a slight, not statistically significant effect, but no synergism was observed with the combined treatments by the test samples and UV. It may be of technical interest that there was considerable variation in the numbers of micronuclei in duplicate cultures of the pigmentary $TiO_2$-exposed cells. This may have resulted from the heterogenicity of the dust sample and/or from difficulties in analysing the cells with high concentrations of $TiO_2$ [42].

In the most often used version of the micronucleus test cytochalasin B is applied in order to inhibit cytokinesis. It may be of technical interest that it was found that cytochalasin B can inhibit the uptake of particulate material into cells [43]. This may contribute to negative outcome of cytochalasin B blocked micronucleus assay with particulate material, although it must be stressed that the micronucleus test was positive in the majority of the reports on genotoxicity tests on nanomaterials located for this review (11 positive, 2 negative, described here below). Papageorgiou et al. [5] observed that a split exposure of first 12 h to nanoparticles alone followed by 12 h combined exposure to cytochalasin B + nanomaterial led to a considerably higher increase of micronuclei compared with a standard combined exposure to cytochalasin B + test material for 24 h. This procedure may help to increase the sensitivity of the micronucleus test with nanoparticles.

After treatment of human lung A549 cells with 1 or 2 μg/cm$^2$ bulk or nanoscaled *vanadium oxides* ($V_2O_3$, $V_2O_5$) for 24 h no induction of micronuclei was observed (K.B. Fischer and H.F. Krug, personal communication and Poster at INIS, Hannover 2008).

Trimethoxysilane modified by rhodamine B isothiocyanate was mixed with tetraethoxysilane, injected into polyvinylpyrolidone-stabilized cobalt ferrite ethanol solution and polymerized on the surface of the cobalt ferrites to form 50 nm *silica-overcoated magnetic nanoparticles labeled with rhodamine B isothiocyanate: MNPs@$SiO_2$(RITC)*. The silica shell thickness was adjusted such that it could be reproduced by controlling the concentration of tetraethoxysilane. All MNPs@$SiO_2$ (RITC) were confirmed by TEM. 0.25, 0.5, and 1 mg MNP@$SiO_2$(RITC) were tested in the chromosome aberration assay in Chinese hamster lung fibroblasts exposed for 6 h using 0.2 μg/mL colcemid to arrest cells in the metaphase. No increase in chromosome aberrations occurred while 0.2 μg/mL mitomycin C used as positive control led to a drastic increase [41].

Warheit et al. [40] used ultrafine *$TiO_2$* consisting of 79% rutile and 21% anatase. A composition of ~90 wt% $TiO_2$, 7% alumina, and 1% amorphous silica was determined by X-ray fluorescence. Dynamic light scattering showed that the median particle size was 140 nm in water (aqueous solution buffered in 0.1% tetrasodium pyrophosphate). The BET surface area was 38.5 m$^2$/g. The particle samples underwent neutralization of acidic chloride groups on the particle surface. In the chromosomal aberration test CHO cells were exposed to 62.5, 125 or 250 μg/mL in sterile water (Milli Q) for 4 h (in the presence of Aroclor-induced rat liver S9) or to 750, 1250 or 2500 μg/mL for 4 h (not activated by S9) or to 25, 50 or 100 μg/mL for 20 h (not activated). None of the treatments gave a positive response (the test substance formed a white but homogeneous suspension at 50 mg/mL, the highest concentration used for preparing stock solutions) [40].

Theogaraj et al. [44] reported that none of 8 different forms of $TiO_2$ was able to induce increases in the frequency of chromosome aberrations in CHO-WBL cells treated with or without UV irradiation while nitroquinoline N-oxide used as positive control without UV irradiation and 8-methoxypsoralene with UV irradiation led to drastic increases. The cells were maintained in McCoy's 5A medium with 10% FCS. The UV irradiation was from a solar simulator (750 mJ/cm$^3$) with a temperature of the irradiated area of 35 °C maintained by a SunCool® unit. Wavelengths <290 nm were removed by a glass filter. The tested materials had the following properties: A, B and C: crystal type anatase 80%, rutile 20%, primary particle size approximately 21 nm as determined by TEM; A: coating trimethoxy caprylylsilane, B: no coating but doped with 2% di-iron trioxide, C: no coating; D: 100% rutile, primary particle size 14 nm as determined by X-ray diffraction, coating 8–11% alumina and 1–3% simethicone; E: 100% anatase, aggregate size 60 nm as determined by X-ray disc centrifugation, coating 37% alumina and 12–18% silica; F-H: 100% rutile, F: primary particle size 20 nm as determined by X-ray diffraction, coating 5–6.5% alumina and 1–4% dimethicone, G: 15 nm primary particle size as determined by TEM, coating 3–8% alumina and 5–11% stearic acid, H: primary particle size 20–22 nm as determined by TEM, coating 10.5–12.5% alumina and 3.5–5% silica. The primary particles rarely exist independently in dispersions. Typically they form aggregates of 30–150 nm. The authors expect such aggregates in the samples tested. For the chromosome aberration test sample A was dissolved in absolute ethanol, B and C in physiological saline, D-H in DMSO. A top concentration which produced without UV irradiation about 50% cell count reduction was chosen or for little toxic samples top concentrations of approximately 5000 μg/mL [44].

#### 3.3.1. Brief overview on Section 3.3

Five studies on Chromosome mutations were negative, all of them performed in vitro. Of these only 2 micronucleus assays were negative (while 12 were positive, see Section 2.3). The negative micronucleus assays were performed on $TiO_2$ [42] and on $V_2O_3$ and $V_2O_5$ (K.B. Fischer and H.F. Krug, personal communication and Poster at INIS, Hannover 2008). Three chromosome aberration tests were negative (while also 3 chromosome aberration tests were positive, see Section 2.3). The negative chromosome aberration tests were performed on silica-overcoated magnetic nanoparticles labeled with rhodamine B isothiocyanate "MNPs@$SiO_2$(RITC)" [41] and on $TiO_2$ [40,44] (Table 1).

## 4. Genotoxicity tests which gave positive versus negative results depending on the particle size

Nanosized (10 and 20 nm) anatase *$TiO_2$* particles sterilized (120°, 2 h) and suspended in sterilized PBS (10 μg/mL) induced in human bronchial epithelial cells (BEAS-2B, cultured in LHC-9

medium containing 10% FCS and ~2 mM glutamate) in the absence of light in the Comet assay with FPG oxidative DNA damage determined as strand breaks and base damage in terms of sites sensitive to FPG as well as an increase in micronuclei formation. However, larger sized material of the same chemical content (anatase titanium dioxide 200 nm or >200 nm in diameter) did not induce any of these DNA damaging events in the absence of light [8].

Nanoparticulate *Carbon Black* Printex 90 (primary diameter 14 nm, suspended at 100 μg/mL in serum-free DMEM and sonicated for 20 min) caused in treated A549 type II alveolar-like human lung adenocarcinoma cell line after 3 h exposure a significant increase in single-strand DNA breaks and alkali-labile sites (alkaline/neutral Comet assay), but coarse carbon black (primary particle diameter 260 nm) did not produce these effects [7].

Nanoparticulate *$TiO_2$* (<20 nm, sterilized by heating to 120 °C for 2 h and suspended at 1 μg/μL in PBS) led in Syrian hamster embryo fibroblasts (cultured in modified Dulbecco's Eagle's reinforced medium supplemented with 15% FCS and 1% glucose) tested at 0.5, 1, 5 and 10 μg/cm$^2$ to a significant ($p \leq 0.05$) increase in micronuclei (24.5–31.3 micronuclei/1000 cells from 12 to 72 h of exposure; controls ~18 to ~23 micronuclei/1000 cells estimated from the figure). In contrast, larger $TiO_2$ particles (>200 nm) did not induce micronuclei to a significant extent [29].

In these three examples the nanosized material was positive in the genotoxicity test, the corresponding larger sized materials were negative. In a fourth example the nanosized material was more genotoxic, but the difference was only quantitative, not qualitative:

Nanoparticles of *cobalt chrome alloy* (mean size: 29.5 ± 6.3 nm; round to oval, determined after coating with 5–10 nm platinum/palladium and then viewed by field emission gun electron microscopy; 62.2% Co, 28.7% Cr, 6.3% Mo, 0.87% Si, 0.71 Ni, 0.59% Mn, 0.53% Fe, 0.057% C, similar to the composition in orthopaedic joint replacement protheses) suspended in MEM and sonicated for 30 s caused in the alkaline Comet assay in primary human dermal fibroblasts after 24 h of exposure DNA damage in a dose-dependent manner (tail moment at highest dose of 5000 μm$^3$/cell, about 17 fold compared with control cells) while larger sized cobalt chrome alloy (mean size: 2.904 ± 1.064 μm) led to considerably less DNA damage (about 4-fold difference at the highest dose), yet the test was still clearly positive also with the larger size material. The higher DNA damaging activity of the nanomaterial compared with the corresponding micromaterial was in accordance with the observed radical formation which was much higher with nanosized cobalt chrome alloy compared with the microsized material, not reaching significance with the latter. Also in the micronucleus assay in primary human dermal fibroblasts (concentrations: 5, 50, 500 μm$^3$/cell; 12 h exposure to CoCr alloy alone followed by 12 h cytochalasin B + CoCr alloy) both materials were positive, but the nanosized material caused more centromer-positive micronuclei, i.e. its aneugenicity was higher [5].

However, not always is nanosized material more genotoxic than corresponding larger sized material. While nanosized *$V_2O_3$* was positive in the Comet assay (on human lung alveolar type II adenocarcinoma cells, concentrations: 1 and 2 μg/cm$^2$, time: 24, 36, 48 h) and bulk sized $V_2O_3$ was negative, the results with $V_2O_5$ were opposite: nanosized *$V_2O_5$* was negative and bulk sized $V_2O_5$ was positive. The potential to generate reactive oxygen species correlated with the relative water solubility, yet the genotoxicity did not: of the four materials the most water soluble, the nano $V_2O_5$ and the least water soluble, the bulk $V_2O_3$ were negative in the Comet assay (K.B. Fischer and H.F. Krug, personal communication and Poster at INIS, Hannover 2008).

## 5. Positive versus negative genotoxicity results depending on the test used

In some studies available in the open scientific literature various genotoxicity tests performed with the same nanomaterial in the same study, i.e. under identical conditions of the preparation of the identical nanomaterial, gave contrasting results in different tests, i.e. apparently really related to the test used.

In a study by Nakagawa et al. [14] nanosized (21 nm) anatase *$TiO_2$* was photogenotoxic in the Comet assay and in the chromosome aberration assay in Chinese hamster lung CHL/IU cells, but no photogenotoxicity was observed in bacterial (Ames) or mammalian cell (mouse lymphoma L5178 tk+/−) gene mutation assays. *Details:* For exposure to UV–vis light from a sunlight simulator (50 min) L5178Y mouse lymphoma cells and $TiO_2$ p-25 particles, anatase form, average size 21 nm were suspended in EBSS. After irradiation the cells were suspended in MEM culture medium with 10% FCS for the alkaline Comet assay which showed a dose-dependent increase in tail length which increased with increasing UV energy from 1.6 to 5 J/cm$^2$. No increase in tail length was seen without irradiation. Chinese hamster CHL/IU cells (suspended in MEM with 10% FCS) were exposed to the same $TiO_2$ P25 particles for 1 h in the dark, and then exposed to the same UV–vis light at 5 J/cm$^2$ for 50 min. After treatment with demecolcin for arrest in metaphase 12.5 to 50 μg/mL $TiO_2$ led to a dose-dependent increase in chromosome aberrations (mostly chromatid breaks and exchanges). No increase in chromosome aberrations was seen without irradiation. The same preparation of $TiO_2$ P25 particles was negative in the Ames Salmonella/Micosome assay at concentrations of 5000, 10,000, 20,000 and 40,000 μg/mL using the strains TA100, TA98 and TA102 with and without the same UV–vis light irradiation (10 min, 1 J/cm$^2$ or 50 min, 5 J/cm$^2$). It was also negative in the mammalian cell mutation assay at concentrations of 250, 500, 750, 1000, 1250, 1500 and 2000 μg/mL with or without UV–vis irradiation for 50 min at 5 J/cm$^2$ using L5178Y mouse lymphoma cells (maintained in RPMI 1640, 1% sodium pyruvate, 0.05% pluronic F-68 and 10% horse serum and for the mouse lymphoma assay suspended in EBSS) [14].

In a report by Wang et al. [24] nanosized *$SiO_2$* was positive in the micronucleus assay (cytokinesis block version) and in the mammalian cell gene mutation assay (HPRT), but negative in the DNA damage Comet assay. *Details:* $SiO_2$ (99% purity) nanoparticles (7.21–123 nm) were suspended in culture medium (RPMI 1640 supplemented with 5% FCS), vortexed for 10 min, then sonicated for 10 min in ultrasonic water bath. In WIL2-NS human B-cell lymphoblastoid cells exposed there was (as determined by the micronucleus assay with cytochalasin B to inhibit cytokinesis) a significant dose-dependent increase in micronucleated binuclear cells with increasing dose of particles (a 4-fold increase compared with untreated cells at 120 μg/mL with the 24-h treatment; the lowest dose that gave a statistically significant increase was 30 μg/mL with 24-h treatment). In the same cells exposed to the same $SiO_2$ nanoparticle preparation (120 μg/mL) for 24 h the mutation frequency detected by HPRT mutation assay was also significantly increased by $3.8 \times 10^{-5}$ (above a background of $6.8 \times 10^{-5}$). However, in the same cells exposed to 30, 60 or 120 μg/mL of the same $SiO_2$ nanoparticle preparation for 6, 24 or 48 h the alkaline Comet assay was negative (no increase in % tail DNA, tail length or olive tail moment while all three parameters were increased after treatment with 0.1 mM $H_2O_2$ as a positive control) [24].

Quite opposite, in the report by Kisin et al. [4] another nanomaterial, *SWCNT*, was positive in the DNA damage Comet assay, practically negative in the micronucleus assay (limited but not statistically significant micronucleus induction) and negative in the bacterial gene mutation Ames test (in the Salmonella strains

YG1024 or YG1029). *Details:* SWCNT with diameters from 0.4 to 1.2 nm, a length of 1–3 μm and a surface area of 1040 $m^2$/g, comprised of 99.7 wt% element carbon and iron levels of approximately 2.3 mg Fe/g (0.23 wt%) were ultrasonicated (30 s × 3 cycles) and investigated by the Comet assay in V79 cells (seeded into MEM supplemented with 10% FCS) at concentrations of 0, 24, 48 or 96 μg/$cm^2$. Already after 3 h of incubation with 96 μg/$cm^2$ of SWCNT the Comet assay showed significant DNA damage (4.2-fold increase of olive tail moment in comparison with vehicle treated cells). The same preparation of the same nanomaterial tested in the same medium at concentrations of 12, 24, 48, or 96 μg/$cm^2$ for 24 h showed limited (<2 fold) but not statistically significant micronucleus induction at the highest concentrations tested (positive control N-methyl-N-nitro-N-nitrosoguanidine). The same preparation of the same nanomaterial was negative in the Ames Salmonella assay at concentrations of 60, 120, or 240 μg/plate using the strains YG1024 and YG1029 without S9 mix [4] (Table 1).

## 6. Apparently surprising positive versus negative results with respect to the substance investigated

In a study reported by Jacobsen et al. [9] nanoparticulate *Carbon Black* (primary size 14 nm) induced a statistically significant increase in DNA strand breaks in the Comet assay while *quarz* (IARC group 1: Carcinogenic in human) (mean particle size 1.59 μm) did not. Also carbon black, but not quartz, weakly increased the mutant frequency in both the *cII* and *lacZ* genes in the FE1 Muta™Mouse lung epithelial cell line. Quite to the contrary a study reported by Zhong et al. [38] found that quarz (α-quarz, <5 μm) resulted in the Comet assay in a significant, concentration-related increase in tail length, but Carbon Black (37 nm) did not. *Details: Study by Jacobsen et al.* [9]: Carbon Black particles (Printex 90, primary particle size: 14 nm, specific surface area: 295 $m^2$/g, organic impurity content ~1% [of the 16 EPA priority polycyclic aromatic hydrocarbons phenanthrene, fluoranthene, pyrene, benzo[*a*]anthracene, and chrysene were detected]) were sonicated and cultured in DMEM F12 (1:1) medium, 2% FCS, 2 mM L-glutamine, 1 ng/mL murine epidermal growth factor. At 75 μg/mL the particles induced within 3 h in the FE1 Muta™Mouse lung epithelial cell line an increase in DNA strand breaks ($p = 0.02$) and oxidized purines ($p = 0.008$) detected in the alkaline Comet Assay with FPG (the authors concluded that the low level of organic impurities is not expected to be causal for the observed DNA strand breaks in the Comet assay). However, quartz (specific surface area: 2.9 $m^2$/g, mean particle size 1.59 μm, preparation of the particles in the same way as for the nanoparticulate carbon black and tested in the Comet assay under the same conditions, but even at a slightly higher concentration: 100 μg/mL) did not induce DNA strand breaks (although it did increase FPG-sensitive sites, but not statistically significant). Mutant Frequencies in the *cII* and *lacZ* genes were investigated in the same FE1 Muta™Mouse lung epithelial cell line with 75 μg/mL Carbon Black and 100 μg/mL quartz. The cells were incubated with the tested compounds in 8 exposure rounds making the total exposure time (8 × 72 h) = 576 h. The cumulative dose added was (8 × 75 μg × 10 mL) = 6 mg for carbon black and (8 × 100 μg × 10 mL) = 8 mg for quartz. The mutant frequency for the Carbon Black treated cells was increased 1.4 fold ($p = 0.0002$) for the *cII* gene and 1.23 fold ($p = 0.002$) for the *lacZ* gene. The change in *cII* and *lacZ* mutant frequency for quartz treated cells was not statistically significant (nominal increase of 1.3 fold for *cII* and decrease of 0.89 fold for *lacZ*). Study by Zhong et al. [38]: Crystalline silica (Min-U-Sil 5, 99% alpha-quartz, <5 μm, autoclaved, sonicated in MEM, vortexed and sonicated again for 30 min) resulted in the alkaline Comet assay in V79 Chinese hamster lung fibroblasts and in Hel 299 human embryonic lung fibroblasts exposed for 3 h to concentrations of 17.2–103.4 μg/$cm^2$ in a significant, concentration-related increase in tail length, but Carbon Black (99% carbon, 37 nm particles) prepared and assayed in the same way was at (the even slightly higher) concentrations from 17.2 to 137.9 μg/$cm^2$ negative [38].

A recent study (K.B. Fischer and H.F. Krug, personal communication and Poster at INIS, Hannover 2008) shows that the same nanomaterial can give discordant results in different genotoxicity tests which (seemingly unpredictably) are discordant in a completely different way for (seemingly) quite closely related materials: Nanosized $V_2O_3$ was positive in the Comet assay (on human lung alveolar type II adenocarcinoma cells, concentrations: 1 and 2 μg/$cm^2$, time: 24, 36, 48 h), while under the same conditions nanosized $V_2O_5$ was negative in the Comet assay. Of 4 investigated materials (nanosized and bulk $V_2O_3$ and $V_2O_5$) the potential to generate reactive oxygen species correlated with the relative water solubility, but not with the response in the Comet assay.

## 7. What can we learn?

### 7.1. Know what nanomaterial has been tested (and in what form)

A first conspicuous observation when considering the reports on nanoparticle genotoxicity investigations available in the open scientific literature was that many experiments used particles not well characterized with respect to chemical composition and physicochemical properties. However, in order to draw valid conclusions from the tests such information is important. Information on the hydrophilicity/hydrophobicity and the charge of the particle surface is essential since these parameters profoundly influence the dispersion of the material in the test medium and this influences the adsorption of proteins and other components of the test medium. In most studies the agglomeration state under the actual test conditions is not communicated and probably not investigated, but is in fact strongly influenced by the test conditions and, to close the circle, it strongly influences surface chemistry.

### 7.2. Consider uptake and distribution of the nanomaterial

Although uptake from the cytosol into the nucleus may not be a prerequisite for interaction of the test material with nuclear DNA since the barrier by the nuclear envelope is not effective during nuclear division, in many cases it is not investigated whether uptake of the test material into the cell does actually take place. A lack of uptake may be a reason for negative tests with, e.g. some bacteria which possess a complicated cell wall.

In the most often used version of the micronucleus test cytochalasin B is applied to inhibit cytokinesis. Cytochalasin B can also inhibit the uptake of particles into cells [43]. This may contribute to negative outcome of cytochalasin B blocked micronucleus assay with particles, although the micronucleus test was positive in the majority of the reports on genotoxicity tests on nanomaterials located for this review (11 positive, 2 negative). Papageorgiou et al. [5] observed that a split exposure of first 12 h to nanoparticles alone followed by 12 h combined exposure to nanomaterial + cytochalasin B led to a considerably higher increase of micronuclei compared with a standard 24 h combined exposure to test material + cytochalasin B. Such a split exposure may increase the sensitivity of the micronucleus test with nanoparticles.

Aerosols from nanomaterials are complex systems and so is the uptake and deposition of inhaled nanomaterial in the lung. Hence,

for inhalation exposure, inhalation studies are preferred over instillation studies and in vitro systems.

### 7.3. Use standardized methods

An obvious field for improvement in any nanomaterial toxicity testing including genotoxicity testing is the difficulty for objective comparisons due to the lack of standardization including differentiated test protocols for nanomaterials, definition of metrics such as mass, surface area and particle number as well as internationally agreed reference nanomaterial. The intelligent (i.e. nanomaterial-appropriate) use of genotoxicity tests which are validated and for which guidelines exist will facilitate the comparability of the obtained results.

### 7.4. Recognize that nanomaterials are not all the same

It is obvious that the similarities among nanomaterials with respect to very small size (nanoscale) and very large surface to mass ratio does not mean that all nanoparticles are similar in respect to other important properties. It is likely that nanotubes, a few nanometers in diameter but microns in length, may in some respects follow rules which have already been established for silicate fibres showing that respirable fibre types fundamentally differ from each other in their ability to cause lung damage including genotoxicity and carcinogenicity depending on the thinness and long needle-like shape of the fibres and their biopersistence in the lung [45]. The length and diameter of the nanotubes are likely to be key players and should therefore be known and given in publications on their genotoxicity testing.

With respect to another, quite different, actually sub-nano class, the fullerenes ($n$C$_{60}$), it appears to have a pronounced effect on genotoxicity testing whether employing aggregates synthesized using intermediate organic solvents or whether employing an aggregate production method that more closely simulates the fate of $n$C$_{60}$ upon accidental release: extended mixing in water [46]. $n$C$_{60}$ produced through several solvent exchange processes or through extended mixing in water only, were unique from each other with respect to size, morphology, charge, and hydrophobicity. The greatest dissimilarities were observed between the $n$C$_{60}$ produced by extended mixing in water alone versus those produced by solvent exchange [47]. Markovic et al. [48] prepared $n$C$_{60}$ suspensions by solvent exchange method in tetrahydrofuran (THF/$n$C$_{60}$) and ethanol (EtOH/$n$C$_{60}$), or by extended mixing in water (aqu/$n$C$_{60}$). The capacity to generate ROS was THF/$n$C$_{60}$ > EtOH/$n$C$_{60}$ > aqu/$n$C$_{60}$. Mathematical modelling of singlet oxygen ($^1$O$_2$) generation indicated that the $^1$O$_2$-quenching (THF/$n$C$_{60}$ < EtOH/$n$C$_{60}$ < aqu/$n$C$_{60}$) of the solvent intercalated in the fullerene crystals determined the production of ROS. Aqu/$n$C60 suspensions elicited considerably higher genotoxic response than EtOH/$n$C60 for the same $n$C60 concentration [3]. Thus, fullerenes mobilized by natural processes (agitation in water) behave dramatically differently compared with those produced through solvent exchange methods highlighting the need for care in extrapolating fullerene properties such as potential toxicities from one preparation to another and perhaps pointing to possibilities for mechanistic considerations also concerning true nanoparticles. However, it should be noted that on grounds of the sub-nano size (<1 nm) $n$C60 should be clearly separated from true nanomaterials, actually not only because its size is technically clearly below the nanometer range, but especially because its properties are very different from those of true nanomaterials.

### 7.5. Use in vivo studies to correlate in vitro results

The reports found in the publically available scientific literature presented in this review show that DNA damage tests (especially the Comet assay) and chromosome mutation assays (especially micronucleus tests) have successfully been used with nanoparticles. These tests, like any new test methods and modified protocols of existing methods have yet to be validated. Given the complex nature of nanomaterial's dispersion in air or liquids (aerosols or suspensions) and the complex process of their uptake, deposition and distribution in the body, in vivo genotoxicity studies have an obvious advantage and, for inhalation exposure, inhalation studies are preferred over instillation studies.

Although requiring great effort, it would be of obvious advantage to have data from whole animal carcinogenicity assays with nanomaterials to improve the basis for genotoxicity tests (in addition to the general correlations with just any material already available to-date). Obviously more easily available than cancer bioassays are observations on occurrence or non-occurrence of hyperplasia, dysplasia or pre-neoplastic lesions in repeated dose toxicity studies which may help to put genotoxicity test results into perspective beside the obvious possibility to pursue positive in vitro tests with nanomaterials by in vivo genotoxicity tests. This may include Comet or micronucleus test in the target tissue (such as the lung if the route of exposure is by inhalation) or in the blood or bone marrow (if the availability of the material at those targets or a wide systemic availability has been shown or is at least likely) using appropriate application routes.

### 7.6. Take nanomaterials specific properties into account

Indeed, the vast majority of the genotoxicity investigations on nanomaterials found in the literature employed generally used genotoxicity testing methods. This is a practical and pragmatic approach which, however, in most cases does not explicitly take into consideration that nanoparticles have properties which make them different from the same basic material with larger particle size. While soluble or biodegradable nanoparticles which disintegrate in organismic or cellular targets into molecular species (liposomes, nanoemulsions) may obey similar laws as their larger sized counterparts or solutions thereof, insoluble or biopersistent nanoparticles such as $TiO_2$ or quantum dots possess distinct properties. Most conspicuously, the nanoparticle's large surface area per unit mass is prone to lead to an increased biological reactivity enhancing any intrinsic toxic response compared with the same mass of the larger sized material with the same chemical content. Moreover, depending on their size and the dispersion agent, nanoparticles will either diffuse within the liquid or sediment onto exposed cells in culture [49]. Due to the large surface per unit mass nanoparticles have a higher adsorption capacity potentially leading to binding of contaminants present during manufacturing and/or present during testing. Carbon nanotubes as hollow cylinders contain metal contaminants used as catalysts during production likely to induce oxidative stress during genotoxicity testing. Also the high adsorption capacity may lead to the particles becoming coated with proteins when introduced in test media [50] depending on particle size, concentration, aggregation and surface [51]. The coating leads to differences in the actual properties of the material primarily due to influences on the effective size and charge of the particle [52]. The adsorption of nutrients and growth factors from culture media [53] could lead to confounding results from genotoxicity tests which depend on cell proliferation. Moreover, the high surface/mass relationship of nanoparticles results in an increase in surface energy enhancing catalytic activities leading in many cases to the

**Table 2**
Recommendations for genotoxicity testing of nanomaterials.

| |
|---|
| Know what nanomaterial has been tested and in what form |
| Recognize that nanomaterials are not all the same |
| Consider uptake and distribution of the nanomaterial |
| Take nanomaterials specific properties into account |
| Use standardized methods |
| Use in vivo studies to correlate in vitro results |
| Learn about the mechanism of nanomaterials genotoxic effects |

production of genotoxic reactive oxygen species (ROS) by nanoparticles [54 and references therein]. Hence, tissue distribution, biopersistance, catalytic activity and inflammation potential (including but not limited to ROS generation) may be decisive factors in nanoparticles genotoxicity (and also general toxicity).

### 7.7. Learn about the mechanism of genotoxic effects

Different nanomaterials are engineered to have various unique material properties. Obviously these properties will also affect their possible direct or indirect interaction with the DNA. Although it has been shown that cationic functionalized carbon nanotubes can condense with DNA (the surface area and positive charge density being considered critical) [55] and that binding of 1.4 nm (but not larger or smaller) gold nanoparticles to the major grove of DNA is associated with killing of cancer cells [56], for most nanomaterials it is even unknown whether they directly interact with DNA or whether indirect effects such as inflammation-mediated oxidative stress may infer a threshold for the genotoxicity of some nanomaterials. Recognizing different ways by which various nanomaterials interact with DNA will improve the possibility for an optimal choice of tests and test conditions and for extrapolations of genotoxicity test results to human risk (Table 2).

## 8. Conclusion

Experiences with other, non-nano, substances (molecules and larger particles) taught us, that mechanisms of genotoxic effects can be diverse and their elucidation can be demanding, while there often is an immediate need to assess the genotoxic hazard. Thus a practical and pragmatic approach is the use of a battery of standard genotoxicity testing methods covering a wide range of mechanisms. Application of these standard methods to nanomaterials demands, however, several adaptations and the interpretation of results from the genotoxicity tests may need additional considerations. This review should help to improve testing of nanomaterials by generally used genotoxicity testing methods as well as investigations on the underlying mechanism and the interpretation of genotoxicity data on nanomaterials.

## Conflict of interest

There is no conflict of interest.

## Acknowledgments

The support by the EU Network of Excellence ECNIS (Environmental Cancer, Nutrition and Individual Susceptibility) operating within the EU 6th Framework Program, Priority 5: "Food Quality and Safety" (Contract no. 513943) and the EU project NanoSafe2 within the EU 6th Framework Program and the German BMBF lead project nanoCare is gratefully acknowledged.

## References

[1] D.B. Warheit, C.M. Sayes, K.L. Reed, K.A. Swain, Health effects related to nanoparticle exposures: environmental, health and safety considerations for assessing hazards and risks, Pharmacol. Ther. 120 (2008) 35–42.
[2] V.L. Colvin, The potential environmental impact of engineered nanomaterials, Nat. Biotechnol. 21 (2003) 1166–1170.
[3] A. Dhawan, J.S. Taurozzi, A.K. Pandey, W. Shan, S.M. Miller, S.A. Hashsham, V.V. Tarabara, Stable colloidal dispersions of C60 fullerenes in water: evidence for genotoxicity, Environ. Sci. Technol. 40 (2006) 7394–7401.
[4] E.R. Kisin, A.R. Murray, M.J. Keane, X.C. Shi, D. Schwegler-Berry, O. Gorelik, S. Arepalli, V. Castranova, W.E. Wallace, V.E. Kagan, A.A. Shvedova, Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells, J. Toxicol. Environ. Health Part A 70 (2007) 2071–2079.
[5] I. Papageorgiou, C. Brown, R. Schins, S. Singh, R. Newson, S. Davis, J. Fisher, E. Ingham, C.P. Case, The effect of nano- and micron-sized particles of cobalt–chromium alloy on human fibroblasts in vitro, Biomaterials 28 (2007) 2946–2958.
[6] J.J. Wang, B.J. Sanderson, H. Wang, Cyto- and genotoxicity of ultrafine $TiO_2$ particles in cultured human lymphoblastoid cells, Mutat. Res. 628 (2007) 99–106.
[7] R.M. Mroz, R.P. Schins, H. Li, L.A. Jimenez, E.M. Drost, A. Holownia, W. MacNee, K. Donaldson, Nanoparticle-driven DNA damage mimics irradiation-related carcinogenesis pathways, Eur. Respir. J. 31 (2008) 241–251.
[8] J.R. Gurr, A.S. Wang, C.H. Chen, K.Y. Jan KY, Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells, Toxicology 213 (2005) 66–73.
[9] N.R. Jacobsen, A.T. Saber, P. White, P. Møller, G. Pojana, U. Vogel, S. Loft, J. Gingerich, L. Soper, G.R. Douglas, H. Wallin, Increased mutant frequency by carbon black, but not quartz, in the lacZ and cII transgenes of muta mouse lung epithelial cells, Environ. Mol. Mutagen. 48 (2007) 451–461.
[10] M. Dybdahl, L. Risom, J. Bornholdt, H. Autrup, S. Loft, H. Wallin, Inflammatory and genotoxic effects of diesel particles in vitro and in vivo, Mutat. Res. 562 (2004) 119–131.
[11] P.S. Vinzents, P. Møller, M. Sørensen, L.E. Knudsen, O. Hertel, F.P. Jensen, B. Schibye, S. Loft, Personal exposure to ultrafine particles and oxidative DNA damage, Environ. Health Perspect. (2005) 1485–1490.
[12] P.H. Avogbe, L. Ayi-Fanou, H. Autrup, S. Loft, B. Fayomi, A. Sanni, P. Vinzents, P. Møller, Ultrafine particulate matter and high-level benzene urban air pollution in relation to oxidative DNA damage, Carcinogenesis 26 (2005) 613–620.
[13] E.V. Bräuner, L. Forchhammer, P. Møller, J. Simonsen, M. Glasius, P. Wåhlin, O. Raaschou-Nielsen, S. Loft, Exposure to ultrafine particles from ambient air and oxidative stress-induced DNA damage, Environ. Health Perspect. 115 (2007) 1177–1182.
[14] Y. Nakagawa, S. Wakuri, K. Sakamoto, N. Tanaka, The photogenotoxicity of titanium dioxide particles, Mutat. Res. 394 (1997) 125–132.
[15] R. Dunford, A. Salinaro, L. Cai, N. Serpone, S. Horikoshi, H. Hidaka, J. Knowland, Chemical oxidation and DNA damage catalysed by inorganic sunscreen ingredients, FEBS Lett. 418 (1997) 87–90.
[16] M. Green, E. Howman, Semiconductor quantum dots and free radical induced DNA nicking, Chem. Commun. 121 (2005) 121–123.
[17] Y. Zheng, D.J. Hunting, P. Ayotte, L. Sanche, Radiosensitization of DNA by gold nanoparticles irradiated with high-energy electrons, Radiat. Res. 169 (2008) 19–27.
[18] Q. Zhang, Y. Kusaka, K. Sato, K. Nakakuki, N. Kohyama, K. Donaldson, Differences in the extent of inflammation caused by intratracheal exposure to three ultrafine metals: role of free radicals, J. Toxicol. Environ. Health Part A 53 (1998) 423–438.
[19] S.S. Leonard, V. Castranova, B.T. Chen, D. Schwegler-Berry, M. Hoover, C. Piacitelli, D.M. Gaughan, Particle size-dependent radical generation from wildland fire smoke, Toxicology 236 (2007) 103–113.
[20] Z. Li, T. Hulderman, R. Salmen, R. Chapman, S.S. Leonard, S.H. Young, A. Shvedova, M.I. Luster, P.P. Simeonova, Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes, Environ. Health Perspect. 115 (2007) 377–382.
[21] G. Chen, J. Zhao, X. Liu, G. Gao, J. Huang, G. Li, Electrochemical sensing DNA damage with nano-titanium dioxide and repair with a medicinal herb species resveratrol, J. Biotechnol. 127 (2007) 653–656.
[22] S. Maenosono, T. Suzuki, S. Saita, Mutagenicity of water-soluble FePt nanoparticles in Ames test, J. Toxicol. Sci. 32 (2007) 575–579.
[23] J.J. Wang, B.J.S. Sanderson, H. Wang, Cytotoxicity and genotoxicity of ultrafine crystalline $SiO_2$ particulate in cultured human lymphoblastoid cells, Environ. Mol. Mutagen. 48 (2007) 151–157.
[24] J.J. Wang, H. Wang, B.J.S. Sanderson, Ultrafine quartz-induced damage in human lymphoblastoid cells in vitro using three genetic damage end-points, Toxicol. Mech. Methods 17 (2007) 223–232.
[25] L. Zhu, D.W. Chang, L. Dai, Y. Hong, DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells, Nano Lett. 7 (2007) 3592–3597.
[26] H.G. Claycamp, Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxyguanine assays, Carcinogenesis 13 (1992) 1289–1292.
[27] E. Driscoll, L.C. Deyo, J.M. Carter, B.W. Howard, G. Hassenbein, T.A. Bertram, Effect of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells, Carcinogenesis 18 (1997) 423–430.
[28] G. Oberdörster, A. Maynard, K. Donaldson, V. Castranova, J. Fitzpatrick, K. Ausman, J. Carter, B. Karn, W. Kreyling, D. Lai, S. Olin, N. Monteiro-Riviere, D. Warheit, H. Yang, Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy, Particle Fibre Toxicol. 2 (2005) 8–43.

[29] Q. Rahman, M. Lohani, E. Dopp, H. Pemsel, L. Jonas, D.G. Weiss, D. Schiffmann, Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in syrian hamster embryo fibroblasts, Environ. Health Perspect. 110 (2002) 797–800.
[30] L. Wang, J. Mao, G.H. Zhang, M.J. Tu, Nano-cerium-element-doped titanium dioxide induces apoptosis of Bel 7402 human hepatoma cells in the presence of visible light, World J. Gastroenterol. 13 (2007) 4011–4014.
[31] E.K. Dufour, T. Kumaravel, G.J. Nohynek, D. Kirkland, H. Toutain, Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells, Mutat. Res. 607 (2006) 215–224.
[32] J. Muller, I. Decordier, P.H. Hoet, N. Lombaert, L. Thomassen, F. Huaux, D. Lison, M. Kirsch-Volders, Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells, Carcinogenesis 29 (2008) 427–433.
[33] M.L.L. Freitas, L.P. Silva, R.B. Azevedo, V.A.P. Garcia, L.M. Lacava, C.K. Grisolia, C.M. Lucci, P.C. Morais, M.F. Da Silva, N. Buske, R. Curi, Z.G.M. Lacava, A double-coated magnetite-based magnetic fluid evaluation by cytometry and genetic tests, J. Magn. Magn. Mater. 252 (2002) 396–398.
[34] N. Sadeghiani, L.S. Barbosa, L.P. Silva, R.B. Azevedo, P.C. Morais, Z.G.M. Lacava, Genotoxicity and inflammatory investigation in mice treated with magnetite nanoparticles surface coated with polyaspartic acid, J. Magn. Magn. Mater. 289 (2005) 466–468.
[35] J.H. Park, K.T. Han, K.J. Eu, J.S. Kim, K.H. Chung, B. Park, G.S. Yang, K.H. Lee, M.H. Cho, Diffusion flame-derived fine particulate matters doped with iron caused genotoxicity in B6C3F1 mice, Toxicol. Ind. Health 21 (2005) 57–65.
[36] H. Hidaka, S. Horikoshi, N. Serpone, J. Knowland, In vitro photochemical damage to DNA. RNA and their bases by an inorganic sunscreen agent on exposure to UVA and UVB radiation, J. Photochem. Photobiol. A: Chem. 111 (1997) 205–213.
[37] M. Auffan, L. Decome, J. Rose, T. Orsiere, M. Demeo, V. Briois, C. Chaneac, L. Olivi, J.L. Berge-Lefranc, A. Botta, M.R. Wiesner, J.Y. Bottero, In vitro interactions between DMSA-coated maghemite nanoparticles and human fibroblasts: a physicochemical and cyto-genotoxical study, Environ. Sci. Technol. 40 (2006) 4367–4373.
[38] B.Z. Zhong, W.Z. Whong, T.M. Ong, Detection of mineral-dust-induced DNA damage in two mammalian cell lines using the alkaline single cell gel/comet assay, Mutat. Res. 393 (1997) 181–187.
[39] B. Rehn, F. Seiler, S. Rehn, J. Bruch, M. Maierd, Investigations on the inflammatory and genotoxic lung effects of two types of titanium dioxide: untreated and surface treated, Toxicol. Appl. Pharmacol. 189 (2003) 84–95.
[40] D.B. Warheit, R.A. Hoke, C. Finlay, E.M. Donner, K.L. Reed, C.M. Sayes, Development of a base set of toxicity tests using ultrafine $TiO_2$ particles as a component of nanoparticle risk management, Toxicol. Lett. 171 (2007) 99–110.
[41] J.S. Kim, T.J. Yoon, K.N. Yu, B.G. Kim, S.J. Park, H.W. Kim, K.H. Lee, S.B. Park, J.K. Lee, M.H. Cho, Toxicity and tissue distribution of magnetic nanoparticles in mice, Toxicol. Sci. 89 (2005) 338–347.
[42] K. Linnainmaa, P. Kivipensas, H. Vainio, Toxicity and cytogenetic studies of ultrafine titanium dioxide in cultured rat liver epithelial cells, Toxicol. in Vitro 11 (1997) 329–335.
[43] D.R. Haynes, S.D. Rogers, D.W. Howie, M.J. Pearcy, B. Vernon-Roberts, Drug inhibition of the macrophage response to metal wear particles in vitro, Clin. Orthop. Relat. Res. 323 (1996) 316–326.
[44] E. Theogaraj, S. Riley, L. Hughes, M. Maier, D. Kirkland, An investigation of the photo-clastogenic potential of ultrafine titanium dioxide particles, Mutat. Res. 634 (2007) 205–219.
[45] K. Donaldson, C.L. Tran CL, An introduction to the short-term toxicology of respirable industrial fibres, Mutat. Res. 553 (2004) 5–9.
[46] L.K. Duncan, J.R. Jinschek, P.J. Vikesland, C60 colloid formation in aqueous systems: effects of preparation method on size, structure, and surface charge, Environ. Sci. Technol. 42 (2008) 173–178.
[47] J.A. Brant, J. Labille, J.Y. Bottero, M.R. Wiesner, Characterizing the impact of preparation method on fullerene cluster structure and chemistry, Langmuir 22 (2006) 3878–3885.
[48] Z. Markovic, B. Todorovic-Markovic, D. Kleut, N. Nikolic, S. Vranjes-Djuric, M. Misirkic, L. Vucicevic, K. Janjetovic, A. Isakovic, L. Harhaji, B. Babic-Stojic, M. Dramicanin, V. Trajkovic, The mechanism of cell-damaging reactive oxygen generation by colloidal fullerenes, Biomaterials 28 (2007) 5437–5448.
[49] L.K. Limbach, Y. Li, R.N. Grass, T.J. Brunner, M.A. Hintermann, M. Muller, D. Gunther, W.J. Stark, Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations, Environ. Sci. Technol. 39 (2005) 9370–9376.
[50] R.S. Kane, A.D. Stroock, Nanobiotechnology: protein–nanomaterial interactions, Biotechnol. Prog. 23 (2007) 316–319.
[51] T. Cedervall, I. Lynch, S. Lindman, T. Berggard, E. Thulin, H. Nilsson, K.A. Dawson, S. Linse, Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 2050–2055.
[52] C. Schulze, A. Kroll, C.M. Lehr, U.F. Schäfer, K. Becker, J. Schnekenburger, C. Schulze Isfort, R. Landsiedel, W. Wohleben, Not ready to use—overcoming pitfalls when dispersing nanoparticles in physiological media, Nanotoxicology 2 (2008) 51–61.
[53] L. Guo, A. Von Dem Bussche, M. Buechner, A. Yan, A.B. Kane, R.H. Hurt, Adsorption of essential micronutrients by carbon nanotubes and the implications for nanotoxicity testing, Small 4 (2008) 721–727.
[54] A. Isakovic, Z. Markovic, B. Todorovic-Markovic, N. Nikolic, S. Vranjes-Djuric, M. Mirkovic, M. Dramicanin, L. Harhaji, N. Raicevic, Z. Nikolic, V. Trajkovic, Distinct cytotoxic mechanisms of pristine versus hydroxylated fullerene, Toxicol. Sci. 91 (2006) 173–183.
[55] R. Singh, D. Panatarotto, D. McCarthy, O. Chaloin, J. Hoebeke, C.D. Partidos, J.P. Briand, M. Prato, A. Bianco, K. Kostarelos, Binding and condenstation of plasmid DNA onto functionalized carbon nanotubes: toward the construction of nanotube-based gene delivery vectors, J. Am. Chem. Soc. 127 (2005) 4388–4396.
[56] Y. Pan, S. Neuss, A. Leifert, M. Fischler, F. Wen, U. Simon, G. Schmid, W. Brandau, W. Jahnen-Dechent, Size-dependent cytotoxicity of gold nanoparticles, Small 3 (2007) 1941–1949.