

**COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT**

**GENOTOXICITY ASSESSMENT OF NANOMATERIALS AND EXPERIMENTAL CONSIDERATIONS**

**INTRODUCTION**

1. Validated genotoxicity assays have been standardised for single chemical compounds and the assessment of nanomaterials using the same test methods has been questioned. Due to their specific physico-chemical properties, nanomaterials tend to have high reactivity and may interact with experimental components of assays with the consequent generation of misleading data. Assay compatibility, therefore, is an important consideration. This discussion paper presents some discrepancies in nanomaterial genotoxicity data and the potential confounding factors in existing standard assays when used for the assessment of nanomaterials. In addition, a broad overview of evidence for DNA-mediated genotoxicity and indirect DNA damage in the presence of nanomaterials has been provided. Members are requested to consider this literature and answer the questions on the genotoxicity testing of nanomaterials at the end of the discussion paper.

**BACKGROUND**

2. Although no internationally harmonised definition has been agreed, a working definition of a nanomaterial can be considered as a material which is particulate at the nanoscale, mobile in its immediate environment, and either a nano-object or nanostructured with a least one external dimension, or an internal structure, <100 nm in size.<sup>1</sup> Nanomaterials can range in shape (spheres, tubes, rods, fibres, cages) and composition (metals, metal oxides, binary metals), and may have surface modifications e.g. dextran, polyethylene glycol, chemical moieties, DNA and proteins for specific applications.<sup>2</sup> Engineered nanomaterials, including nanoparticles and nanofibres, are categorised into four classes: carbon-based materials, metal-based materials (quantum dots, nanosilver, nanogold), dendrimers (nanosized polymers), and composites.<sup>3</sup> Currently, there are over 1300 consumer products containing nanomaterials on the market (August 2011)<sup>4</sup> and this is set to increase dramatically in coming years.

3. There is considerable uncertainty about the safety of nanomaterials. Due to their physico-chemical characteristics they may have unpredictable genotoxic potential and induce adverse biological effects, and the suitability of standardised genotoxicological assays designed for screening single compounds and the validity of published data have been questioned.<sup>2,5-7</sup> Given the reactivity of nanomaterials, assay compatibility is an important consideration, which if not given sufficient credence, could have a dramatic impact on the validity of resultant datasets.<sup>2</sup> In addition, inconsistencies in the

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literature in terms of dose metrics and limited physico-chemical characterisation means that it can be impossible to compare studies.<sup>6</sup>

4. There is international interest in the development of a battery of standard *in-vitro* tests suitable for the risk assessment of nanomaterials. The Organisation for Economic Cooperation and Development (OECD) has established the Working Party on Manufactured Nanomaterials (WPMN) that has the review of current test guidelines for the applicability to manufactured nanomaterials as one of its objectives. In a preliminary report on OECD test guidelines, the WPMN advises the bacterial reverse mutation assay (OECD TG 471), *in-vitro* mammalian chromosome aberration test, and *in-vitro* mammalian cell gene mutation test (OECD TG 476) should be performed for an initial investigation of mutagenic potential of nanomaterials.<sup>8</sup> The WPMN has selected a set of representative manufactured nanomaterials for use in its safety testing programme: C60 fullerenes, single- and multi-walled carbon nanotubes (SWCNTs and MWCNTs), silver, gold and iron nanoparticles, titanium and silicon dioxides, aluminium oxide, cerium oxide, zinc oxide, dendrimers and nanoclays.<sup>9</sup>

5. Some researchers have suggested adaptations to standard protocols and testing strategies to account for a number of different factors that appear to be involved in modulating biomolecular interactions of nanoparticles, and thereby develop valid genotoxicity assays for their assessment. These suggestions have included the requirement for detailed physico-chemical characterisation; characterisation of aggregation / agglomeration *in-vitro*; inclusion of appropriate experimental controls; determination of the influence of surface modification; assessment of cellular uptake and distribution; correlation between *in-vitro* and *in-vivo* results; and elucidation of a complete view of their genotoxic mechanisms.<sup>5-7,10,11</sup>

## GENOTOXICITY DATA FOR NANOMATERIALS

6. Only limited *in-vitro* genotoxicity data is available for nanomaterials and a number of *in-vitro* studies have been summarised in the following tables according to the assay used.

*Table 1 Ames Test (Bacterial Reversion Mutation Test) / Mammalian Gene Mutation Tests*

Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
SWCNT	0.4-1.2 nm in diameter, 1-3µm in length	Ames salmonella assay using strains YG1024 and YG1029 without S9 mix	Ames negative	12
FePt nanoparticles capped with 2-aminoethanethiol	3 nm	Ames assay using <i>Salmonella typhimurium</i> strains TA98, TA100, TA1535 and TA1537, and <i>Escherichia coli</i> strain	Ames negative	13

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Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
		WP2uvrA/pKM101, with and without metabolic activation by S9 mix in the pre-incubation method (8 h pre-incubation)		
FePt nanoparticles capped with tetramethylammonium hydroxide	9 nm Water-soluble	Ames assay using <i>Salmonella typhimurium</i> strains TA98, TA100, TA1535 and TA1537, and <i>Escherichia coli</i> strain WP2uvrA/pKM101, with and without metabolic activation by S9 mix in the pre-incubation method (8 h pre-incubation)	Weakly positive in the TA100 strain without S9 mix (maximum specific activity was 61.6 revertants/mg), but negative in other cases	14
Silica-overcoated magnetic nanoparticles labelled with rhodamine B isothiocyanate	50 nm	Ames assay using <i>Salmonella typhimurium</i> strains TA97, TA98, TA100, TA102 with and without metabolic activation by S9 mix	Ames negative	15
Titanium dioxide	21 nm	Ames assay using <i>Salmonella typhimurium</i> strains TA98, TA100, TA102 ± irradiation Hypoxanthine guanine phosphoribosyltransferase (HPRT) mutation assay using L5178Y mouse lymphoma cells ± irradiation	Ames negative  HPRT mutation negative	16
Titanium dioxide	Ultrafine particles 79% rutile, 21% anatase median size 140 nm	Chinese hamster CHO-WBL cells ± Aroclor-induced rat liver S9	Ames negative	17
Zinc oxide	Particle size <200 nm. Mean particle size 100 nm	Ames assay using <i>Salmonella typhimurium</i> strains TA98, TA100, TA1537 and <i>E. coli</i> strain WP2	Ames negative	18
Silicon dioxide	Size range 7-123 nm	HPRT mutation assay using WIL2-NS human lymphoblastoid B-cells	HPRT mutation positive	19, 20
Titanium dioxide	Size by volume 6.57, by intensity distribution 8.2 nm (80.4%) – 196.5 nm (19.4%) 99% purity	HPRT mutation assay using WIL2-NS human lymphoblastoid B-cells	HPRT mutation positive	21
Titanium dioxide	21 nm	HPRT mutation assay using L5178Y mouse lymphoma cells with or without UV-vis	HPRT mutation negative	16

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Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
		irradiation		
MWCNT	Size unspecified	Adenine phosphoribosyl-transferase (Aprt) mutation assay using Aprt-heterozygous mouse (C3H/Hej) embryonic stem cells	Aprt mutation positive	22
Carbon black Printex 90	14 nm. Phenanthrene, fluoranthene, pyrene, benzo(a)anthracene and chrysene were present as impurities at approximately 1%	Fe1 Muta™ Mouse lung epithelial cell line.	Weakly genotoxic at the <i>lacZ</i> and <i>cII</i> transgene loci. Study authors concluded that the increase in mutant frequency (1.4 fold for <i>cII</i> and 1.23 for <i>lacZ</i> compared to untreated cells) was unlikely to be due to their presence but might be caused by DNA damage induced during the chloroform/ phenol extraction	23

7. The Ames Test (Bacterial Reversion Mutation Test) may be unsuitable for accurately detecting genotoxicity in nanomaterials because these prokaryotes lack the ability to perform endocytosis and the nanomaterials may be unable to diffuse across the bacterial cell wall.<sup>6</sup> In addition, some nanomaterials, such as silver, are antibacterial.<sup>6</sup> Given concern over the applicability of the Ames test for nanomaterials, it has been suggested by some researchers that the *hprt* or *tk* forward mutation assays utilising mammalian cells may be a more relevant experimental system.<sup>6</sup>

*Table 2 In-vitro Chromosome Aberration Test*

<b>Nanomaterial</b>	<b>Nanoparticle Characteristics</b>	<b>Test System</b>	<b>Results</b>	<b>Ref No</b>
FePt nanoparticles capped with 2-aminoethanethiol	3 nm	Chromosome aberration assay in Chinese hamster lung CHL-IU fibroblasts	Equivocal	13
Silica-overcoated magnetic nanoparticles labelled with rhodamine B isothiocyanate	50 nm	Chromosome aberration assay in Chinese hamster lung fibroblasts	No increase in chromosome aberrations	15
Silver	46 ± 21 nm	Human mesenchymal stem cells in a medium containing 10% fetal calf serum (FCS)	Chromosome aberrations consisting of deletions and exchanges	24
Titanium dioxide P25	21 nm	Chinese hamster lung CHL/IU fibroblasts	Dose-dependent increase in chromosome aberrations, mostly chromatid breaks and exchanges, only after UV-vis irradiation	16
Titanium dioxide	Ultrafine particles 79% rutile, 21% anatase median size 140 nm	Chinese hamster ovary CHO-WBL cells ± Aroclor-induced rat liver S9	No increase in chromosome aberrations	17
Titanium dioxide	Different forms of TiO <sub>2</sub> – ultrafine, crystalline, coated and uncoated	Chinese hamster CHO-WBL cells	None of the forms induced increases of chromosome aberrations	25
Zinc oxide	100 nm, purity >99%, uncoated	Chinese hamster ovary (CHO) cells in a medium containing 10% FCS. Around 1.5 h before harvest the cells were treated with 1 µg/ml colchicines.	The chromosome aberrations, mostly chromatid deletions and exchanges, were concentration related and became statistically significant at 105 µg/ml	18

*Table 3 Comet Assay (Single-Cell Gel Electrophoresis Assay)*

<b>Nanomaterial</b>	<b>Nanoparticle Characteristics</b>	<b>Test System</b>	<b>Results</b>	<b>Ref No</b>
Carbon Black Printex 90	14 nm	Alkaline/neutral comet assay using A549 human adenocarcinoma cells in a serum-free medium	Induced a significant increase in single-strand, but not double-strand, DNA breaks and alkali-labile sites after 3h exposure	26
Carbon Black Printex 90	14 nm	FE1 Muta™ Mouse lung epithelial cells, cultured in a medium containing 2% FCS with and without FPG	Induced a significant increase in DNA strand breaks and oxidised purines within 3h	27
Carbon Black	37 nm, purity 99% carbon	Chinese hamster lung V79 fibroblasts and in He1 299 human embryonic lung fibroblasts	Comet negative	28
C60 Fullerenes	colloidal	Human lymphocytes	Strong correlation between nC60 concentration and genotoxicity with concentrations down to 2.2 µg/L and 4.2 µg/L for aqueous and ethanol suspensions respectively. The Olive tail moments (OTM) for these concentrations were statistically significant compared to the negative control.	29
C60 Fullerenes		FE1 Muta™ Mouse lung epithelial cells, cultured in a medium containing 2% FCS with and without FPG	Comet negative. Significantly increased the level of FPG sensitive sites/oxidized purines by 22%	30
SWCNT	0.4-1.2 nm in diameter, 1-3 µm in length composed of 99.7% carbon and 0.23% iron by weight	Chinese hamster lung fibroblast V79 cells seeded into a medium containing 10% FCS	After 3 h incubation, the highest concentration of SWCNT (96 µg/cm <sup>2</sup> ) showed a 4.2-fold increase of OTM above the controls	12
SWCNT	0.7-1.2 nm in diameter, 0.5-100 µm in length 96.7% carbon, 1.5% Co	Alkaline murine assay in murine macrophage cell line RAW 264.7	Comet positive Oxidized purines increased significantly, whereas pyrimidines showed a significant increase (P < 0.001) only at the highest concentration (100 µg/ml)	31
SWCNT	1.1 nm in diameter, 50 µm in length composed of 96% carbon	Alkaline comet assay in human peripheral blood lymphocytes cultured in a medium containing 15% FCS	Comet negative	32
SWCNT	Average diameter 1.4 nm, 2-5 µm in length 70-90% purity	Normal and malignant human mesothelial cells cultured in a medium containing 5% FCS exposed to 25 or 50 µg/cm <sup>2</sup> SWCNTs for	Exposure of NM cells to 25 or 50 µg/cm <sup>2</sup> SWCNTs resulted in a 5.2- and 6.6-fold increase in DNA tail length migration. Reactive oxygen species (ROS)	33

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Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
		24 h	scavengers only moderately reduced DNA damage	
SWCNT	1-4 nm in diameter, 1-3 $\mu\text{m}$ in length composed of >99.7% carbon and 0.23% iron by weight	Chinese hamster lung fibroblast V79 cells seeded into a medium containing 10% fetal calf serum (FCS).	After 24 h incubation, SWCNT (48 $\mu\text{g}/\text{cm}^2$ ) showed a 3-fold increase of OTM above the controls	34
SWCNT	2-5 $\mu\text{m}$ in length composed of 72% carbon	FE1 Muta™ Mouse lung epithelial cells, cultured in a medium containing 2% FCS with and without FPG	Comet negative. Significantly increased the level of FPG sensitive sites/oxidized purines by 56%, respectively. No effect on mutant frequency in <i>cII</i> gene	30
SWCNT and other CNTs	>50% were SWCNTs and approx. 40% other CNTs  1.1 nm in diameter, 0.5-100 $\mu\text{m}$ in length	Alkaline comet assay in human bronchial epithelial BEAS-2B cells exposed for 24, 48 and 72 h  Conc range 3.8-380 $\mu\text{g}/\text{ml}$	CNTs induced a dose-dependent increase in DNA damage at all treatment times, with a statistically significant effect starting at the lowest dose tested.	35
MWCNT	110-170 nm in diameter, 5-9 $\mu\text{m}$ in length >98% carbon	Alkaline comet assay in murine macrophage cell line RAW 264.7	Comet positive. Increase in DNA migration due to the oxidative damage to purines was observed at a concentration of 1 and 10 $\mu\text{g}/\text{ml}$ , whereas pyrimidines showed a significant increase only at the highest mass concentration tested (100 $\mu\text{g}/\text{ml}$ )	31
Cobalt-chromium alloy	Similar in composition to orthopaedic joint replacement prostheses, mean size $29.5 \pm 6.3$ nm; 62.2% Co, 28.7% Cr, and 0.1% other metallic elements	Human fibroblasts at concentrations of 0.0005-5000 $\mu\text{m}^3$ in a medium containing 10% FCS	Genotoxic response was dose dependent. After 24 h exposure, the highest dose showed a 17-fold increase in tail moment above the controls. Study authors noted that after 3 days exposure the level of DNA damage was much smaller, albeit still significant.	36
Cobalt-nano and $\text{Co}^{2+}$	Co-nano 20-500nm Purity not detailed	Balb/3T3 mouse fibroblasts cultured in a medium containing 5% FCS exposed to Co-nano and $\text{Co}^{2+}$ for 2 h at 1, 3, 5 $\mu\text{M}$	Statistically significant induction of DNA single- and double-strand breaks for both $\text{Co}^{2+}$ (dose-dependent) and Co-nano (not dose-dependent).	37
Maghemite (nano- $\gamma\text{Fe}_2\text{O}_3$ ) coated with DMSA	Mean diameter 6 nm. Negative surface charge barrier to prevent aggregation	Fibroblasts from infant foreskin	Comet negative. Negative surface charge possibly responsible for the negative outcome of the comet assay	38
Nickel, cobalt and titanium	Nickel (20 nm), cobalt (20 nm) and titanium dioxide (28 nm)	Incubated with $\phi\text{X174RF1}$ DNA in buffer (pH 7.2) for 8 h and analysed using the	Showed depletion of supercoiled DNA by 70-75% with either cobalt or nickel nanoparticles, and by	39

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Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
		comet assay	15% with titanium dioxide nanoparticles	
Silicon carbide	5 nanoparticles with varying diameter and Si/C ratio Diameter range 13-68 nm Si/C ratio 0.8-1.3	A549 human lung carcinoma CCL-185 cells cultured in a medium containing 10% FCS with 24 h exposure	Comet positive Induced DNA strand breakages. Si/C-NPs entered nuclei and arranged in clusters 20-30 nm diameter	40
Silicon dioxide	16, 40 and 104 nm, amorphous	Alkaline comet assay in human lung carcinoma A549 cells in a medium containing 10% FCS	Weak and not statistically significant induction of oxidative DNA damage, chromosome breakage and chromosome loss	41
Silicon dioxide	Size range 7-123 nm	Alkaline comet assay in WIL2-NS human lymphoblastoid B-cells exposed for 24 h	Comet negative	19, 20
Silicon dioxide	30, 80 & 400 nm Nanoparticles were purified and their dispersion characteristics in experimental solution conditions characterised before testing	Alkaline comet assay in 3T3-L1 fibroblasts with 3, 6, and 24 h incubations and 4 & 40 µg/ml of silica nanoparticles. Results were independently validated in two separate laboratories	Comet negative	42
Silicon dioxide	50 ± 3 nm Doped with luminescent dyes	Human lung A549 epithelial cells in a medium containing 10% newborn bovine serum exposed for 48 and 72 h	Comet negative. Nanoparticles enter cells but not nuclei	43
Silver	6-20 nm starch coated	Human lung fibroblasts IMR-90 and human glioblastoma cells U251 exposed for 24 h cultured in a medium containing 15% FCS	Comet positive. Concentration-dependent increase in tail momentum was more pronounced in cancer cells	44
Silver	46 ± 21 nm	Human mesenchymal stem cells in a medium containing 10% FCS	Comet positive. Concentration-dependent increase in %Tail DNA. At 24 h, significant increase in DNA fragmentation (P<0.05) compared to 1 & 3 h at conc 1-10 µg/ml.	24
Titanium dioxide	Size by volume 6.57, by intensity distribution 8.2 nm (80.4%) – 196.5 nm (19.4%) 99% purity	Alkaline comet assay using WIL2-NS human lymphoblastoid B-cells in a medium containing 5% FCS	After 24 h, treatment with 65 µg/ml TiO <sub>2</sub> nanoparticles induced a 3-fold increase in %Tail DNA and 5-fold increase in OTM.	21
Titanium dioxide	10 and 20 nm	Human bronchial epithelial BEAS-2B cells cultured in a medium containing 10% FCS in total darkness ± formamidopyrimidine	DNA strand breaks and base-damage	45

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Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
		glycosylase		
Titanium dioxide P25	21 nm	Alkaline comet assay in L5178Y mouse lymphoma cells	Comet positive after irradiation	16
Titanium dioxide	25 nm 8:2 anatase:rutile	Alkaline/neutral comet assay using human embryo L-02 hepatocytes cultured in a medium containing 10% FCS and exposed to 0.01, 0.1 and 1 µg/ml for 24 h	Comet negative	46
Titanium dioxide	30 nm 70-85% anatase & 30-15% rutile	Alkaline comet assay using human peripheral blood lymphocytes cultured in a medium with 10% FCS exposed for 6, 12 or 24 h	Comet positive Reduced effect when cells were pre-treated with N-acetyl-cysteine	47
Titanium dioxide	<100 nm	Alkaline comet assay in human lung IMR-90 fibroblasts and human bronchial epithelial BEAS-2B cells cultured in a medium containing 10% FCS	Comet negative. Significant DNA adduct formation in IMR-90 cells (8-OHdG)	48
Titanium dioxide	Nanoparticles 90-110 nm & bulk particles	Human lymphocytes	Nanoparticles showed significant DNA damage (%Tail DNA) at lowest conc 0.25 mM followed by a gradual decrease. Bulk particles induced a dose-dependent increase in DNA damage (P≤0.05) at conc >1 mM.	49
ZnO	Mean size 30 nm	Alkaline comet assay using human epidermal A431 cells cultured in a medium containing 10% FCS and exposed to 0.001-5 µg/ml ZnO for 6 h	Comet positive. 4-fold increase in OTM at highest dose.	50
ZnO	30 and 165 nm	Alkaline comet assay in human liver HepG2 cells cultured in a medium containing 10% FCS and exposed to ZnO for 6 h	Comet positive. Significant increase (P<0.05) in OTM	51

*Table 4 Micronucleus Test*

<b>Nanomaterial</b>	<b>Nanoparticle Characteristics</b>	<b>Test System</b>	<b>Results</b>	<b>Ref No</b>
Aluminium oxide	28 ± 19 nm	Chinese hamster ovary CHO-K1 cells exposed for 24 h, co-treatment with cytochalasin-B	Dose-dependent increase in number of micronuclei	52
C60 Fullerenes	7.1 ± 2.4 nm	Chinese hamster ovary (CHO) cells, human epidermoid-like carcinoma (HeLa) cells and human embryonic kidney 293 (HEK293) cells cultured in a medium containing 2% FCS. Absence of cytochalasin-B. Exposure period 33-80 days	At 33 days, HEK293 and HeLa cells showed increased cell proliferation. At 80 days, all cell types induced increase in number of micronuclei	53
SWCNT	0.4-1.2 nm in diameter, 1-3 µm in length composed of 99.7% carbon and 0.23% iron by weight	Chinese hamster lung fibroblast V79 cells incubated in a medium without fetal calf serum (FCS).	After 24 h incubation, the highest concentration of SWCNT (96 µg/cm <sup>2</sup> ) showed no significant increase in DNA damage	12
SWCNT	0.7-1.2 nm in diameter, 0.5-100 µm in length 96.7% carbon, 1.5% Co	Murine macrophage cell line RAW 264.7 cultured in a medium containing 10% FCS with post-treatment with cytochalasin-B 20 h after the addition of MWCNT to culture	Increase in number of micronuclei at doses above 0.1 µg/ml (P < 0.05).	31
SWCNT	1-2 nm in diameter, 400-800 nm in length 98% purity and surface area of 585 m <sup>2</sup> /g	Human bronchial epithelial BEAS-2B cells cultured in a medium containing either 2% or 10% FCS for 48 h and either post- or co-treatment, or absence of cytochalasin-B.	Micronucleus induction varied between assays. See paragraph 34	2
SWCNT	1-4 nm in diameter, 1-3 µm in length composed of 99.7% carbon and 0.23% iron by weight	Chinese hamster lung fibroblast V79 cells incubated in a medium without both FCS and cytochalasin-B treatment.	After 24 h incubation, SWCNT (12 µg/cm <sup>2</sup> ) showed significant (1.9-fold) micronucleus induction	34
SWCNT	Dimensions not stated 70% purity functionalised with amides	Human lymphocytes cultured in a medium containing 10% FCS treated with 24 h delayed co-treatment with cytochalasin-B.  Enumeration of gamma H2AX foci as a measure of double strand breaks of the DNA in normal human	Increase in micronucleus induction in both cell types.  In the fibroblasts there was a 2.7-fold increase in gamma H2AX foci above the control	54

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Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
		dermal fibroblasts HDMEC.		
SWCNT & other CNTs	>50% were SWCNTs and approx. 40% other CNTs  1.1 nm in diameter, 0.5-100 µm in length	Human bronchial epithelial BEAS-2B cells treated for 24, 48 and 72 with cytochalasin-B co-treatment. Absence of serum in medium. 3.8-380 µg/ml	Equivocal	35
MWCNT	11.3 nm in diameter, 0.7 µm in length 98% carbon with traces of cobalt and iron catalysts	Rat liver epithelial (RLE) cells suspended in a medium containing 5% FCS and MCF-7 breast cancer cells in medium containing 10% FCS were exposed separately. Post-treatment with cytochalasin-B.	There was a significant increase in micronuclei, up to 2-fold at the cytotoxic dose of 50 µg/ml, in RLE epithelial cells, and centromere-positive and negative micronuclei were produced in the MCF-7 cells.	55
MWCNT	20-40 nm in diameter, 1-5 µm in length 99% purity	Human lymphocytes cultured in a medium containing 10% FCS treated with 24 h delayed co-treatment with cytochalasin-B.  Enumeration of gamma H2AX foci as a measure of double strand breaks of the DNA in normal human dermal fibroblasts HDMEC	Induced lymphocyte micronuclei and anaphase bridges among nuclei in binucleated cells. Acted as a clastogen and aneugen simultaneously	54
MWCNT	110-170 nm in diameter, 5-9 µm in length >98% carbon	Murine macrophage cell line RAW 264.7 cultured in a medium containing 10% FCS with post-treatment with cytochalasin-B 20 h after the addition of MWCNT to culture	Increase in number of micronuclei at doses above 1 µg/ml (P < 0.05).	31
Cobalt-chromium alloy	Similar in composition to orthopaedic joint replacement prostheses, mean size 29.5 ± 6.3 nm; 62.2.% Co, 28.7% Cr, and 0.1% other metallic elements	Human fibroblasts cultured in a medium containing 10% FCS. Micronuclei determined using the micronucleus test, 12 h exposure to nanoparticle followed by 12 h exposure to cytochalasin-B	Induced a significant (p<0.001) and dose-dependent increase in micronuclei. Both centromere-positive and negative micronuclei were produced. Particles accumulated in the cytoplasm of the cells and at higher doses they tended to obscure the micronuclei but not the fluorescent comets of the fragmented nuclei	36
Cobalt-nano and Co <sup>2+</sup>	Co-nano 20-500 nm Purity not detailed	Balb/3T3 mouse fibroblasts cultured in a medium containing 5%	Statistically significant (P<0.001) induction of micronuclei for Co-nano,	37

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Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
		FCS exposed to Co-nano and Co <sup>2+</sup> for 24 h at 1, 3, 5 µM. Post-treatment with cytochalasin-B.	but not dose-dependent. No genotoxic effect observed for Co <sup>2+</sup> .	
Silicon dioxide	16, 60 and 104 nm, amorphous	Human lung carcinoma A549 cells in a medium containing 10% FCS exposed for 40 h with delayed treatment with cytochalasin-B	No statistical genotoxic effect	41
Silicon dioxide	Size range 7-123 nm	WIL2-NS human B-cell lymphoblastoid cells in a medium containing 5% FCS with cytochalasin-B	Induced a 4-fold increase in micronucleated binuclear cells at 120 µg/ml after 24 h. The lowest dose to produce a significant increase was 30 µg/ml with 24 h treatment	19, 20
Silver	6-20 nm starch coated	Human lung fibroblasts IMR-90 and human glioblastoma cells U251 cultured in a medium containing 15% FCS exposed for 24 h, delayed post-treatment with cytochalasin-B	Increase in number of micronuclei	44
Titanium dioxide	Size by volume 6.57, by intensity distribution 8.2 nm (80.4%) – 196.5 nm (19.4%) 99% purity	Cytokinesis block micronucleus assay with WIL2-NS human lymphoblastoid B-cells in a medium containing 5% FCS	After 6 h, treatment induced a significant (P<0.01) increase in micronucleated binuclear cells at 26, 65 & 130 µg/ml	21
Titanium dioxide	10 and 20 nm	Human bronchial epithelial BEAS-2B cells cultured in a medium containing 10% FCS with cytochalasin-B co-treatment in darkness	Exposure to 10 µg/ml TiO <sub>2</sub> for 24 h in total darkness showed a 2.5-fold increase in micronuclei formation	45
Titanium dioxide	<20 nm Physico-chemical characterisation not reported	Syrian hamster embryo fibroblasts cultured in medium containing 15% FCS	Significant increase in micronuclei (P≤0.05) from 12 – 72 h exposure. Kinetichore-positive micronuclei were only insignificantly increased indicating clastogenic but no aneugenic activity	56
Titanium dioxide	20 ± 7 nm	Chinese hamster ovary CHO-K1 cells cultured in a medium containing 10% FCS exposed for 24 h, co-treatment with cytochalasin-B	Increase in number of micronuclei at 0.5 and 1.0 µg/ml	52
Titanium dioxide	25 nm 8:2 anatase:rutile	Human embryo L-02 hepatocytes cultured in a medium containing 10% FCS and exposed to 0.01, 0.1 and 1 µg/ml	No induction of micronuclei	46

Nanomaterial	Nanoparticle Characteristics	Test System	Results	Ref No
		for 24 h, post-treatment with cytochalasin-B		
Titanium dioxide	30 nm 70-85% anatase & 30-15% rutile	Human peripheral blood lymphocytes cultured in a medium with 10% FCS exposed for 48 h with delayed co-treatment with cytochalasin-B	Significantly increased micronucleus formation and DNA breakage. DNA damage caused by ROS generation	47
Titanium dioxide P25 and UV-TITAN M60	P25: ~20 nm, uncoated anatase UV-TITAN M160: ~20 nm, rutile coated with aluminium hydroxide and stearic acid, washed before exposure to remove stearic acid	Cytokinesis block micronucleus assay in RLE cells cultured in a medium containing 20% FCS. After 1h of incubation, half of the slides were irradiated with a UV lamp. All cultures treated with cytochalasin-B and incubated for further 20h.	No increase in the number of micronucleated cells.	57

## Discrepancies in Genotoxicity Data

### Substance

8. The same or related nanomaterials have given different results in the same genotoxicity tests. For example, Carbon Black (14 nm) but not quartz (mean size 1.59  $\mu\text{m}$ ) induced a statistically significant increase in DNA strand breaks in the comet assay and weakly increased the mutant frequency of both *cI* and *lacZ* genes in the FE1 Muta™ Mouse lung epithelial cell line.<sup>27</sup> Conversely, quartz ( $\alpha$ -quartz, <5  $\mu\text{m}$ ) but not Carbon Black (37 nm) induced a significant concentration-dependent increase in tail length in Chinese hamster lung fibroblasts (V79 cells) and human embryonic lung fibroblasts (HEL 299 cells) (Table 3).<sup>28</sup>

9. Also, in one study C60 fullerenes were found not to induce significant increases in DNA strand breakages in FE1 Muta™ Mouse lung epithelial cell line as detected by the comet assay<sup>30</sup> whereas comet positive responses were seen in human lymphocytes in another study<sup>29</sup> (Table 3). Furthermore, C60 fullerenes were found to induce micronuclei in Chinese hamster ovary (CHO) cells, human epidermoid-like carcinoma (HeLa) cells and human embryonic kidney 293 (HEK293) cells (Table 4).<sup>53</sup>

### Particle size

10. Six studies have compared directly the genotoxicity of nanosized and the corresponding larger sized materials (Tables 2, 3 and 4). In four of the studies, the nanosized but not the larger sized materials gave a positive outcome in genotoxicity tests. Titanium dioxide (10 and 20 nm) induced oxidative DNA damage in human bronchial epithelial cells (BEAS-2B) in the comet assay and an increase in micronuclei formation (Tables 3 and 4).<sup>45</sup> By contrast, titanium dioxide 200 nm or >200 nm in diameter did not induce any of these DNA changes.<sup>26</sup> Nanoparticulate Carbon Black Printex 90 (14 nm),

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but not coarse carbon black, induced single strand breaks and alkali-labile sites in Alkaline/neutral comet assay using A549 human adenocarcinoma cells (Table 3).<sup>26</sup> Titanium dioxide nanoparticles (<20 nm), but not particles >200 nm, induced a significant increase in micronuclei in Syrian hamster embryo fibroblasts (Table 4).<sup>56</sup> Statistically significant results were seen with cobalt nanoparticles (20-500 nm) in the micronucleus test and comet assay, whereas for Co<sup>2+</sup> micron-sized particles positive results were observed only with the latter assay.<sup>37</sup>

11. In a fifth study, the nanomaterial was quantitatively more genotoxic than the larger sized material. Cobalt chromium alloy nanoparticles (29.5±6.3 nm) induced dose-dependent DNA damage in primary human dermal fibroblasts in the comet assay, whereas micron-sized cobalt chromium alloy particles (2.904±1.064 µm) resulted in less DNA damage with around a 4-fold difference at the highest dose.<sup>36</sup> Furthermore, in the micronucleus assay in human dermal fibroblasts both the nano- and micron-sized particles induced a dose-dependent increase in gross chromosomal damage that was largely aneugenic. The nanosized material induced more centromere-positive micronuclei than the micron-sized material (Tables 3 and 4). It has been suggested by Singh and co-workers that the discrepancy between the assay results could be due to differences in incubation time as particles may require lengthier treatment times due to their size and mechanism of cellular uptake.<sup>6</sup> In addition, as the degree of nanoparticle agglomeration was unreported, so the true size of the particles exposed to the cells was unknown.<sup>6</sup>

12. In the remaining study using amorphous silica nanoparticles (16, 40 and 104 nm), the smallest particles showed a slightly higher fold induction of micronuclei at non-cytotoxic doses.<sup>41</sup> When considering the three nanoparticles together, the study authors considered that particle number and total surface area appeared to account for micronuclei induction as both correlated significantly with the amplitude of the effect and that using nominal or cellular dose did not show statistically significant differences.

#### *Test*

13. Some studies performed with the same material have given contrasting results in different studies. For example, nanosized silicon dioxide (7-123 nm) was positive in the micronucleus assay with cytochalasin-B and in the HPRT mutation assay but negative in the comet assay (Tables 1, 3 and 4).<sup>20,42</sup> Also, SWCNTs (0.4–1.2 nm, length 1-3 µm) were Ames negative, produced limited but not statistically significant micronucleus induction and were comet positive (Tables 1, 3 and 4).<sup>12</sup> Furthermore, titanium dioxide nanoparticles were negative in the Ames test and chromosome aberration assay but positive in the comet and micronucleus assays.<sup>17,47</sup>

## **CONFOUNDING FACTORS INFLUENCING GENOTOXICITY STUDIES**

14. A number of factors might be involved in modulating the biomolecular interactions of nanomaterials or lead to a misinterpretation of the test results.

## **Size and Morphology of Nanoparticles**

15. Their reduced size may increase their likelihood of crossing biological barriers and govern their kinetics including absorption, distribution, metabolism and excretion. Within the body, nanomaterials may be sufficiently small to readily enter cells through diffusion or endocytosis and promote interactions with biomolecules which may have a negative effect on human health.<sup>6</sup> Inhalation studies have demonstrated that nanoparticles penetrate deeper into the lungs, become localised within many cell types, induce a greater inflammatory response and are often associated with increased toxicity as compared to fine sized particles.<sup>6</sup> Given the established relationship between chronic inflammation, DNA damage and carcinogenesis, this evidence suggests a cause for concern with regards to the long-term health implications following nanoparticle inhalation.<sup>6</sup>

16. The size and morphology of nanoparticles influence internalisation and are key determinants in the surface area associated with a given mass dose.<sup>6</sup> A larger surface area enhances reactive potential and catalytic activity. Therefore, if these nanomaterials are able to enter cells, there is an increased chance that the enhanced surface area of nanoparticles may result in biomolecular interactions, cellular damage and oxidative stress.<sup>6</sup> One study focusing on the toxicological relationship associated with morphology has demonstrated that removal of structural defects from the carbon framework of MWCNT substantially reduced their inflammatory response and overall toxicity.<sup>55</sup>

## **Impurities**

17. Metal impurities in the nanomaterial introduced during synthesis may be responsible for genotoxicological responses. Post-production purification of nanomaterials may still result in 15% residual metal by mass in the final product and may damage the nanomaterial. For example, the purification of carbon nanotubes involving washing or ultrasonification with dilute acid can introduce defects and chemically modify their surfaces.<sup>58-60</sup> It has been suggested that the inclusion of metal chelators in *in-vitro* studies could assist in determining the role of metal impurities.<sup>6</sup>

## **Surface Characteristics**

18. Surface characteristics of nanomaterials can influence their behaviour under different experimental conditions.<sup>6</sup> Surface charge and chemistry govern agglomeration and cellular uptake, and may be associated with potential for DNA interaction.<sup>6</sup> Modification of surface chemistry in order to attach other biomolecules such as peptides for cell targeting can impact upon the toxicity of the resultant nanomaterial.<sup>6</sup> For example, studies of quantum dots with various coatings showed that toxicity was highest with an organic coating, followed by amine groups, and least with carboxyl coatings.<sup>6</sup> The effect of surface characteristics on genotoxicity of nanoparticles has not been studied.

## **Dosimetry**

19. The behaviour of nanoparticles in cell culture media is a function of systemic and particle properties, which in turn determine the rate of transport of nanoparticles to cells in culture and cellular dose. There is an issue regarding exposure of cells in *in-vitro* systems and the need to characterise the effective dose, which might represent only a small fraction of the nominal dose.<sup>61</sup> A study has assessed the genotoxic potential of amorphous silica nanoparticles of three different sizes (16, 60 and 104 nm) using A549 lung carcinoma cells in the MN assay. At non-cytotoxic doses, the smallest particles showed a slightly higher fold induction of micronuclei. Overall, particle number and total surface area appeared to account for micronuclei induction as both correlated significantly with the amplitude of the effect. Using nominal or cellular dose did not show statistically significant differences. Likewise, alkaline comet assay and FISH-centromeric probing of the micronuclei indicated a weak and not statistically significant induction of oxidative DNA damage, chromosome breakage and chromosome loss.<sup>41</sup>

20. The OECD has issued a guidance document on sample preparation and dosimetry and has advised that while dosimetry should always report mass concentration, for water insoluble nanomaterials it may be better expressed as a function of surface area or particle number, and so size distribution and surface area measurements would need to be undertaken for each dose.<sup>62</sup>

## ***In-vitro* Culture Medium Components**

21. Nanomaterials may interact with culture medium components. SWCNT have been shown to interact with pH indicators and riboflavin in growth medium thereby reducing nutrient availability in the cell cultures in which they were dispersed. Through alteration of the cell culture medium, SWCNT have been shown to induce an indirect cytotoxicity which may potentially result in a false positive toxic effect being observed in cytotoxicity studies.<sup>63</sup>

## **Agglomeration of Nanoparticles**

22. Generally, nanomaterials tend to form agglomerates >100 nm in size in solution under physiological and experimental conditions which may have significant effects on their toxicity.<sup>64</sup> Furthermore, the addition of serum to cell culture media can affect the agglomeration or surface chemistry of nanoparticles.<sup>64</sup> The OECD has given guidance on the use of saline solution or culture medium to disperse nanomaterials in *in-vitro* test systems.<sup>62</sup> It has advised that dispersal in a protein like BSA is likely to change the balance of protein in a culture containing FCS very little due to its zwitterionic nature, and so aid in prevention of false positive toxicity associated with nutrient depletion resulting from nutrient adsorption onto the nanoparticle surfaces. The OECD advises use of high quality or chemically defined media as the content of serum and BSA can vary between batches. It has been suggested that

characterisation of nanomaterials in solution needs to be undertaken prior to *in-vitro* studies.<sup>64</sup>

23. Both the use of surfactants and modification of surface functionalisation to increase nanomaterial hydrophilicity might also result in an altered genotoxic responses.<sup>6</sup> For example, surface modification of CNT to aid solubilisation has resulted in reduced genotoxicity and altered agglomeration behaviour, and CNT stabilised with a surfactant has been found in some studies to be more cytotoxic.<sup>59,60</sup> However, no comparison was made between the effects of functionalised versus non-functionalised CNTs to determine if the cytotoxic responses were solely due to the surface chemistry.<sup>6</sup> In addition, no published studies have considered genotoxicity changes resulting from the use of surfactants or functionalisation.<sup>6</sup> It has been suggested that although functionalised nanomaterials may be directly used in consumer or clinical applications, the use of surfactants may be more difficult to justify as the agglomerated form may be more representative of true physiological exposure scenarios, with exception of respiratory tract fluid in the lungs.<sup>6</sup> Characterisation of the level of agglomeration under experimental conditions tends to be unreported in published studies.<sup>6</sup>

### **Exposure Time**

24. Traditionally, *in-vitro* genotoxicity tests have an exposure period no more than 24 h as chemical compounds readily diffuse into the cells and their half-life often limits their period of activity.<sup>6</sup> However, the uptake and movement of nanomaterials through cells may be slower than chemical diffusion.<sup>6</sup> In addition, if these materials linger in the cell during mitosis there is an increased chance that they may interact directly with DNA when the nuclear membrane breaks down, or with components of the mitotic machinery thereby potentially disrupting chromosomal segregation. Thus, longer treatment times may be required before a genotoxic effect of a nanomaterial is seen in a test system.<sup>6</sup> Increased particle uptake with extended incubation time has been observed by Colognato and colleagues who reported that cobalt nanoparticle uptake after 48 h was on average double that of uptake measured at 24 h.<sup>65</sup> The long-term fate of nanomaterials once inside cells has also not been addressed experimentally. It has been suggested that although a metallic nanoparticle may not itself induce DNA damage, but if internalised, over time may corrode to release metallic ions that do induce genotoxicity.<sup>6</sup> Alternatively, if a nanomaterials surface is functionalised to aid its biocompatibility, the stability of those surfaces over time must be considered and if it does eventually breakdown, then the genotoxic responses may be significantly different from initial short-term exposure datasets leading to false negative results.<sup>6</sup>

### **Cytochalasin-B Treatment**

25. When using the cytokinesis block micronucleus assay, it has been suggested that the potential interactions of the nanoparticles with cytochalasin-B should also be considered.<sup>5,10</sup>

## **Artifacts**

26. With the comet assay, particles or aggregates can localise at or near comet appearances and affect their quantification due to their fluorescence or ability to quench DNA-staining agents such as ethidium bromide.<sup>10</sup> Furthermore, during the final steps of the comet assay, nanoparticles may come into direct contact with the nuclear DNA and induce artificial damage.<sup>10</sup> It remains to be tested whether artifacts may occur in the micronucleus assay.<sup>10</sup>

## **Experimental Controls**

27. It has been suggested that relevant (positive) nanoparticle controls should be included in the comet assay and micronucleus test, and testing should be performed in the appropriate dose-response range and in relation to cytotoxicity evaluation.<sup>10</sup> Alongside the recommended assay-specific non-particulate positive controls, respirable crystalline silica may be considered a relevant particle control.<sup>10</sup> Other important aspects are nanoparticle dispersion, sonication and cell-type selection.<sup>10</sup>

## **COMPARISON OF MICRONUCLEUS TEST PROTOCOLS**

28. A recent review has examined the applicability of the current OECD micronucleus assay guideline for testing nanomaterials.<sup>7</sup> The review considered 21 published studies where the *in-vitro* micronucleus assay has been applied for the genotoxicity testing of nanomaterials and comparison made of the different protocols used. The individual studies investigated the genotoxicity of a number of nanomaterials: aluminium oxide, carbon nanomaterials, cobalt, iron oxide, silicon and titanium oxide. The studies were evaluated based on chemical composition, particle size and the assay protocols used in terms of cell type, exposure protocol, cytochalasin-B concentration and treatment regime, and the percentage of serum.

29. It has been suggested that the sensitivity of the *in-vitro* micronucleus test for clastogenic and aneugenic events, and its predictivity for *in-vivo* micronucleus assays, might depend on the use of a cell type relevant to the tissue targeted *in-vivo*, and the protocol (with/without cytochalasin-B depending on p53 status and background frequency of binucleates in the cell line).<sup>66</sup>

## **Exposure Time**

30. The majority of studies exposed cells to nanomaterials for at least 24 h with 5 studies using shorter exposures of 3, 6 or 12 h.<sup>7</sup> A 12 h exposure of Syrian hamster embryo fibroblasts to titanium dioxide nanoparticles (<20 nm) did not induce a significant increase of MN. Jin and colleagues showed that penetration of luminescent silica nanoparticles into cells varied with cell type and was time-dependent.<sup>43</sup> The luminescent silica nanoparticles were taken

up more readily by rat alveolar macrophages than lung epithelial A549 cells and were completely taken into A549 cells within 6 h and remained in the cells for 72 h. The luminescent particles did not enter the cell nuclei.

### **Cytochalasin-B Treatment**

31. The different studies used 4 different cytochalasin-B treatment regimes: simultaneous treatment with nanomaterials, treatment after exposure to nanomaterials, addition of cytochalasin-B sometime after nanomaterial treatment, and absence of cytochalasin-B. The assay relying on cytochalasin-B to inhibit cytokinesis, the CBMN assay, tends to be preferred as it limits scoring to only those cells that have undergone mitosis in the presence of the test compound.<sup>2</sup> Cytochalasin also inhibits endocytosis which is an important cell uptake mechanism for some nanomaterials.<sup>2</sup> Comparison has been made of data generated following exposure of MCL-5 human lymphoblastoid B-cells to dextran-coated USPIO for 24 h in the presence of 1% serum with either A) simultaneous exposure of USPIO and cytochalasin-B, B) sequential exposure of USPIO and cytochalasin-B, and C) mononuclear micronucleus test.<sup>2</sup> The same approach was repeated using BEAS-2B lung epithelial cells exposed to synthesised SWCNT (400-800 nm in length, 1-2 nm in diameter, 98% purity and surface area of 585m<sup>2</sup>/g) for 48 h in the presence of 2% serum with dispersal of the SWCNT in the tissue culture media by sonication for 1 h at 4°C.<sup>2</sup> For dextran-coated USPIO, a significant dose-dependent increase in micronuclei was only observed when methodology (B) above was used. There was no increase in frequency of micronucleus induction over a wide dose range with methodology (A), and the authors suggested that this might be due inhibition of nanoparticle endocytosis. The mononuclear micronucleus assay also failed to detect any increase in genotoxicity and the study authors considered that this might be due to the poor sensitivity of the assay in terms of the number of cells needed to be scored.

32. For the SWCNT, significant increases in micronucleus frequency were observed using methodology (A), simultaneous exposure of nanoparticle with cytochalasin-B, which the study authors suggested indicated endocytosis as not the only means of SWCNT leading to DNA damage. The same frequency of micronuclei was observed at the two highest doses using methodology (A) indicating possible saturation, whereas versions (B) and (C) of the methodology gave a dose-dependent increase.<sup>2</sup> The study authors concluded that endocytosis might therefore be partially involved. The frequency of micronuclei observed in the mononuclear assay was approximately half that in the sequential CBMN assay, considered by the study authors as being due to the lower statistical sensitivity of the test.<sup>2</sup>

33. Papageorgiou and colleagues observed with exposure of human fibroblasts to cobalt-chromium alloy nanoparticles, that a split exposure of the first 12 h to nanoparticles alone followed by 12 h combined exposure to cytochalasin-B plus nanomaterial led to a considerably higher increase of micronuclei compared with a standard combined exposure to cytochalasin-B plus test material for 24 h.<sup>36</sup>

## **Serum Content**

34. Serum content in cell culture medium has been indicated to influence nanomaterial uptake into cells but is both cell type and nanomaterial dependent.<sup>2</sup> For example, serum has been shown to be responsible for decreasing the cellular uptake of silica-coated nanoparticles and anionic maghemite nanoparticles in HeLa cells, but promotes the uptake of the latter particles by macrophages.<sup>2</sup> Doak and colleagues found that 10% versus 1% serum in the culture medium reduced the cellular uptake of dextran-coated ultrafine superparamagnetic iron oxide nanoparticles (USPION) in MCL-5 cells.<sup>2</sup> The negative effect of serum on nanoparticle uptake has been suggested to result from increased hydrodynamic diameter or altered electrostatic charges following adsorption of the serum proteins onto the surface of the nanomaterials.<sup>2</sup>

35. Most of the studies evaluated by Gonzalez and co-workers performed the *in-vitro* micronucleus assay with 10% serum.<sup>7</sup> Doak and colleagues found that the presence of 10% as opposed to 2% serum in the culture medium during exposure of BEAS-2B cells treated with SWCNTs substantially reduced the level of micronuclei induction.<sup>2</sup> By contrast, one of the studies evaluated was undertaken in the absence of serum.<sup>35</sup> In that study, BEAS-2B cells were treated with carbon nanotubes (>50% SWCNTs and approximately 40% other nanotubes) and graphite nanofibres with no significant induction of micronuclei.

36. Serum also acts as a surfactant to disperse nanomaterials and this has been reported for a range of nanomaterials including SWCNT, metals and metal oxide nanoparticles.<sup>2</sup> Agglomerated dextran-coated UPSION nanoparticles have been observed to have a lower hydrodynamic diameter in medium containing 10% serum compared to 1% serum which has been attributed to destabilisation of the aggregates by the serum proteins.<sup>2</sup> However, with an increase in serum concentration, the aggregate size increases and the degree of dispersal caused by 1% versus 10% serum becomes less pronounced.<sup>2</sup> It has been suggested, therefore, that size measurements at a single concentration provides insufficient information on the experimental dynamics of nanomaterial agglomeration.<sup>2</sup>

37. MWCNT (outer diameter 11.3 nm, length 700 nm, aggregate hydrodynamic diameter ~1  $\mu$ m) 98% carbon with traces of cobalt and iron were evaluated separately with RLE epithelial cells in a medium containing 5% FCS and MCF-7 cells in a medium with 10% FCS. There was a 2-fold increase of micronuclei in RLE epithelial cells at the cytotoxic dose of 50  $\mu$ g/ml determined using the MN assay with cytochalasin-B. Centromere-positive and -negative micronuclei were produced in MCF-7 cells providing evidence for induction of clastogenic and aneugenic effects by MWCNT.<sup>55</sup>

38. As some nanomaterials do not penetrate the nuclear membrane and may not, therefore, come into contact with the chromatin, it has been suggested by Gonzalez and colleagues that it is critical for cells in the presence of nanoparticles to undergo mitosis during the treatment period to

allow contact with chromatin as disintegration of the nuclear membrane commences.<sup>7</sup>

## **GENOTOXICITY OF NANOMATERIALS**

39. For most nanoparticles it is unknown whether they interact directly with DNA or whether indirect effects such as inflammation-mediated oxidative stress may infer a threshold for the genotoxicity of some nanoparticles.<sup>5</sup> A broad overview of the available literature is provided, with particular emphasis on identifying potential direct DNA interactions.

### **Evidence for DNA-Mediated Genotoxicity**

40. For this mechanism to operate, particles need to enter the nucleus and interact directly with DNA, or cellular constituents that guide chromosome segregation during cell division.<sup>11</sup> Studies have shown that nanoparticles may be taken up not only through endocytosis but by diffusion or adhesive interactions.<sup>67</sup> Studies have suggested that nanoparticles may enter the nucleus<sup>40,68</sup> and mitochondria<sup>69,70</sup> and disturb their function, and may diffuse freely within cells when not bound to membranes presenting potential for them to enter the nucleus and interact with DNA nanoparticles (~50 nm) have been shown to penetrate *E.coli* cells and bind strongly to DNA in a dose-dependent manner, inducing DNA aggregation and inhibiting cell division during log phase.<sup>71</sup> Nuclear penetration has been demonstrated with several types of nanoparticles often specifically designed or surface modified with the aim of targeting cancer cells, introducing therapeutics or for diagnostic imaging.<sup>11</sup>

41. Gold nanoparticles (1.4 nm) without special surface functionalisation were transported into the nucleus of metastatic melanoma cells (MV3 and BLM) with 24.5% of the total internalised nanoparticles becoming bound to DNA.<sup>72</sup> In another study, 25% of internalised citrate-capped gold nanoparticles (5 nm) without surface functionalisation entered the nucleus of HeLa cells.<sup>73</sup> When these gold nanoparticles were functionalised with a nuclear penetrating peptide, SV40 large T antigen, the percentage of internalised nanoparticles entering the nucleus doubled. A systematic study of the cytotoxicity of water-soluble gold nanoparticles stabilised by triphenylphosphine derivatives ranging from 0.8 – 15 nm has been undertaken in four cell lines: connective tissue fibroblasts, epithelial cells, macrophages and melanoma cells.<sup>74,75</sup> The cellular response was size dependent in that 1.4 nm gold particles caused predominantly rapid cell death by necrosis within 12 h while closely related particles 1.2 nm in diameter induced predominantly programmed cell death by apoptosis. Cytotoxicity seen with 1.4 nm gold nanoparticles was associated with oxidative stress, endogenous ROS production, and depletion of the intracellular antioxidant pool. In contrast, gold particles 15 nm in size and Tauredon® (gold thiomalate) were non-toxic up to 60-fold and 100-fold higher concentrations respectively. In another study, 16 nm citrate-capped gold nanoparticles were shown to enter human fibroblast HeLa cells and become trapped in endosomes. Successful nuclear targeting of the fibroblast cells was achieved using surface modification of the

gold nanoparticles with a peptide acting as a nuclear localisation sequence combined with membrane penetrating peptides.<sup>76</sup> In another study, gold nanoparticles (30 nm) conjugated with cell surface and nuclear localisation peptides were transported into the nuclei of human oral squamous carcinoma cells, caused DNA double-strand breaks, and induced cytokinesis arrest where binucleate cell formation occurs at the late stages of mitosis.<sup>77</sup> Cytokinesis arrest led to apoptosis in the cancer cells.<sup>77</sup>

42. Exposure of human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) to starch-coated silver nanoparticles 6-20 nm in size resulted in cell cycle arrest at G<sub>2</sub>/M phase without massive apoptosis or necrosis.<sup>44</sup> The silver nanoparticles were present within the mitochondria and nucleus and the study authors considered this observation an implication of their direct involvement in mitochondrial respiratory chain disruption and DNA damage leading to cell cycle arrest. Comet and CBMN assays demonstrated extensive DNA damage in both cell lines.

43. A study has shown that negatively charged CdTe and CdSe/ZnO quantum dots penetrate human macrophages through endocytosis, becoming enclosed in endosome-like vesicles.<sup>78</sup> The CdTe quantum dots 2-3 nm in size were able to rapidly enter the nucleus via nuclear pore complexes and penetrate nucleoli of the macrophages where they targeted the positively charged histones.<sup>78</sup> In another study, CdTe quantum dots induced re-organisation of nuclear DNA, nuclear shrinkage, chromatin condensation, loss of mitochondrial cristae and global acetylation of histone-3 in human breast epithelial adenocarcinoma (MCF-7) cells treated for 24 h.<sup>79</sup> The CdTe quantum dots induced an increase in the mRNA levels of p-53 target genes, Bax, Puma and Noxa.<sup>79</sup> Quantum dot treatment also induced an increase in overall p53 levels, p53 phosphorylation and its inter-organelle translocation in the cancer cells.<sup>79</sup>

44. A study has shown the size-dependent entry of silica nanoparticles into the nucleus with resultant disturbance to nuclear organisation.<sup>68</sup> Silica nanoparticles sized between 40-70 nm translocated the nucleus and induced topoisomerase I in interphase nuclei to rearrange from being homogeneously distributed throughout the nucleoplasm and concentrated at the nucleolus in interphase nuclei into aberrant nucleoplasmic clusters.<sup>68</sup> The silica nanoparticle-induced aggregates contained nuclear proteins such as CREB binding protein that are essential for gene expression, cellular polyQ proteins and proteasomes.<sup>68</sup> The intranuclear protein aggregates could lead to inhibition of replication, transcription and cell proliferation.<sup>68</sup> Treatment of different cell types, nasal epithelial, lung epithelial, and neuronal cells from human and rodent origin with silica nanoparticles resulted in the formation of aberrant nucleoplasmic clusters in all cell lines treated.<sup>68</sup> The nuclei of airway and neuronal epithelial cells were identified as primary targets of silica nanoparticles.<sup>68</sup> Silica nanoparticles have been found to induce chromosomal damage using the micronucleus assay but not the comet assay (paragraph 12).

45. C60 fullerenes have been shown to be taken up into human monocyte-derived macrophages and sequestered at several sites within the cell

including the cytoplasm, lysosomes, and the nucleus.<sup>80</sup> There are conflicting reports in the literature on the genotoxicity of C60 fullerenes (paragraph 9).

### **Evidence for Indirect DNA Damage**

46. DNA damage may arise through indirect mechanisms where the nanomaterial interacts with cellular proteins rather than DNA itself. Nanomaterials may also induce oxidative stress, inflammation and aberrant cell signalling responses leading to genotoxicity.<sup>6</sup> Oxidative stress usually results from increased intracellular reactive oxygen species and decreased antioxidants. The ROS can react unfavourably with DNA, proteins and lipids.<sup>6</sup> Oxidative DNA attack has been shown by many studies to govern the genotoxic effects of nanoparticles.

47. Exposure of embryonic lung fibroblasts to 20 nm gold nanoparticles at 25 µg/ml resulted in formation of 8-hydroxydeoxy guanosine adducts accompanied by decreased expression of DNA repair genes and cell cycle checkpoint genes MAD2, cyclin B1 and cyclin B2.<sup>81</sup>

48. Cobalt-chromium alloy nanoparticles have been shown to induce chromosomal damage that was largely aneuploidic (paragraph 11).<sup>36</sup>

49. There are conflicting reports on the genotoxicity of titanium dioxide nanoparticles. Several studies in various cell types report DNA strand breakages and point mutations, adduct formation (8-hydroxydeoxyguanosine), chromosomal damage and increases in micronuclei resulting from oxidative stress both *in-vitro* (Tables 3 and 4)<sup>21,45,47-49,52,56</sup> and *in-vivo*<sup>82</sup>. The increase in micronuclei has been demonstrated to result from a clastogenic mode of action.<sup>56,82</sup> However, Hackenberg and colleagues found that titanium dioxide nanoparticles entered the nuclei of human nasal epithelial cells but no cyto- or genotoxic effects were observed.<sup>83</sup> The study authors noted that the nanoparticles tended to aggregate in suspension. Theogaraj and colleagues found eight different forms of titanium oxide particles (anatase TiO<sub>2</sub> (14-21 nm) with various coatings) to be non-genotoxic using the chromosomal aberration test in CHO-K1 cells either in the presence or absence of UV light with 3 h exposure.<sup>25</sup> Also, Warheit and colleagues did not show genotoxicity with titanium dioxide at concentration range 65-250 µg/ml in CHO-K1 cells after 4 h exposure.<sup>17</sup> However, these exposure periods might be too short to detect genotoxic effects as Lu and Zhu and their respective colleagues reported increased micronuclei formation with different types of titanium dioxide nanoparticles after 24 h exposure at concentrations of 0.4 µg/ml and 25-325 µg/ml respectively.<sup>84,85</sup> The cellular responses elicited by these nanoparticles are highly dependent on size and form and in many studies this degree of information is lacking making study and results comparison difficult.<sup>6</sup> It has been proposed that the distinct patterns of genotoxicity seen with titanium dioxide bulk- and nano-particles may be due to agglomeration of the particles (Table 3).<sup>49</sup>

50. SWCNT have been shown to penetrate human cells and become localised in the nucleus.<sup>86</sup> There are conflicting observations on the

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genotoxicity of SWCNT. Studies have reported either some or no induction of DNA strand breakages or increases over background mutation frequencies in the comet assay (Table 3)<sup>12,30,32-35</sup> and micronucleus test (Table 4)<sup>12,31,34,35,54</sup> in different cell types. MWCNT have been shown to induce a significant (2-fold increase at highest dose of 2 mg) and dose-dependent increase in micronuclei in rat pneumocytes after a single intra-tracheal administration.<sup>55</sup> *In-vitro*, MWCNT have been shown to induce increases in chromosomal damage, through both clastogenic and aneugenic events, and point mutations in different cell types (Tables 1, 3 and 4).<sup>22,31,54,55</sup> It has been suggested that different sensitivities of the cell lines or the use of different protocols might explain these conflicting results.<sup>7</sup>

## **DISCUSSION**

51. The available literature presents studies varying widely in their methods, cell lines, nanoparticle size and surface groups, doses, time points, degree of nanoparticle characterisation, nanoparticle agglomeration state under actual test conditions, and information on nanoparticle uptake into cells. The Committee is asked to consider the following questions:

1. Do standard genotoxicity tests offer a practical and pragmatic approach to the genotoxicity testing of nanomaterials given that they may have properties which make them different from the same basic material with larger particle size? What additional considerations, if any, would the Committee recommend with regard to the application of these tests to nanomaterials and the interpretation of test results?
2. Does the evidence for direct DNA interaction of a number of nanomaterials suggest potential generic mechanisms? What further studies on mode of genotoxic action should be considered?
3. Can the Committee recommend particular areas for investigation to improve genotoxicity testing of nanomaterials.
4. What approaches to risk assessment of genotoxicity would the COM advocate for nanomaterials?

**Secretariat**

**August 2011**

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