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**Evaluation of in vitro methods for human hazard assessment applied in the OECD
Testing Programme for the Safety of Manufactured Nanomaterials**

**Series on the Safety of Manufactured Nanomaterials
No. 85**

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Series on the Safety of Manufactured Nanomaterials

No. 85

**EVALUATION OF IN VITRO METHODS FOR HUMAN
HAZARD ASSESSMENT APPLIED IN THE OECD TESTING
PROGRAMME FOR THE SAFETY OF MANUFACTURED
NANOMATERIALS**

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- Nos. 44-54, These items are the dossiers derived from the Testing Programme on Manufactured Nanomaterials which are located at:
<http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm>
- No.55, *Harmonized Tiered Approach to Measure and Assess the Potential Exposure to Airborne Emissions of Engineered Nano-objects and their Agglomerates and Aggregates at Workplaces (2015)*
- No.56, *Analysis of the Survey on Available Methods and Models for Assessing Exposure to Manufactured Nanomaterials (2015)*
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FOREWORD

The OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (the Joint Meeting) held a Special Session on the Potential Implications of Manufactured Nanomaterials for Human Health and Environmental Safety (June 2005). This was the first opportunity for OECD member countries, together with observers and invited experts, to begin to identify human health and environmental safety related aspects of manufactured nanomaterials. The scope of this session was intended to address the chemicals sector.

As a follow-up, the Joint Meeting decided to hold a Workshop on the Safety of Manufactured Nanomaterials in December 2005, in Washington, D.C. The main objective was to determine the “state of the art” for the safety assessment of manufactured nanomaterials with a particular focus on identifying future needs for risk assessment within a regulatory context.

Based on the conclusions and recommendations of the Workshop [ENV/JM/MONO(2006)19] it was recognised as essential to ensure the efficient assessment of manufactured nanomaterials so as to avoid adverse effects from the use of these materials in the short, medium and longer term. With this in mind, the OECD Council established the OECD Working Party on Manufactured Nanomaterials (WPMN) as a subsidiary body of the OECD Chemicals Committee in September 2006. This programme concentrates on human health and environmental safety implications of manufactured nanomaterials (limited mainly to the chemicals sector), and aims to ensure that the approach to hazard, exposure and risk assessment is of a high, science-based, and internationally harmonised standard. It promotes international co-operation on the human health and environmental safety of manufactured nanomaterials, and involves the safety testing and risk assessment of manufactured nanomaterials.

This project, which was approved for inclusion in the WPMN work plan, was led by the Joint Research Centre (JRC) and the International Council for Animal Protection in OECD Programmes (ICAPO). The purpose of this evaluation was to review the dossiers of the Testing Programme and to evaluate which of the existing *in vitro* OECD Test Guidelines (TGs) were used, what other non-guideline methods were applied, and what were the potential limitations of each assay used for testing manufactured nanomaterials (MNMs).

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, pesticides and Biotechnology of the OECD.

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1. INTRODUCTION

1. The Testing Programme for the Testing of Manufactured Nanomaterials (further referred to as “the Testing Programme”) tested 11 manufactured nanomaterials (MNMs) to generate information including their physico-chemical properties, environmental fate, and environmental and mammalian toxicity. The Testing Programme was concluded in March 2013, and the publication of the dossiers via the Organization for Economic Cooperation and Development (OECD) website (<http://www.oecd.org/science/nanosafety/>) started in early 2015. As indicated in the OECD's Guidance manual for the testing of MNMs [ENV/JM/MONO (2009)20/REV], after conclusion of the Testing Programme, the next step is to consider “the status, need for, and coordination of further test development”. (OECD, 2010_[1])
2. The European Commission's Joint Research Centre (JRC), the International Council for Animal Protection in OECD Programmes (ICAPO), and contributors from Italy and United States (U.S.) performed an initial detailed evaluation of the applicability of the *in vitro* toxicity test methods used to determine the human hazard of different types of MNMs in the Testing Programme. The assays/methods were thoroughly evaluated to identify potential next steps related to methods applied to testing of MNMs.
3. The OECD Council recommended in 2013 that in the testing of manufactured nanomaterials, the OECD Test Guidelines (TGs) should be applied, adapted as appropriate to take into account the specific properties of manufactured nanomaterials. Most of the current OECD TGs have not yet been adapted (or are currently in the process) to accommodate for testing of nanomaterials (OECD, 2013_[2]). The aims of the project were to (1) review which OECD Test Guidelines (hereafter TGs) and non-TG *in vitro* assays were applied in the OECD Testing Programme; (2) verify, where possible, to what extent these assays were applicable for MNMs testing; (3) recognise limitations of each assay when used for MNM testing; and (4) identify issues that might need to be further addressed (e.g. by proposing modification to existing TGs). The present report summarises the information extracted from dossiers, presents the evaluation of the information extracted from the dossiers, and identifies potential next steps (e.g. where modification of existing TGs, or proposals of new TGs or Guidance Documents (GDs) could be appropriate). The recommendations and observations presented in this report are only based on the information extracted from the dossiers and recent publications on specific TGs.
4. Certain commercial products or equipment are described in this paper in order to specify adequately the experimental procedures discussed. In no case does such identification imply recommendation or endorsement by any of the jurisdictions or organizations involved in drafting this document nor by the OECD or its Member Countries, nor does it imply that it is necessarily the best available for the purpose.

2. METHODOLOGY

5. The following two-step approach was applied to review and evaluate the available data.
- In the first step, a “screening” of the available dossiers was performed to make a compendium of all the *in vitro* test methods that have been used for testing of each MNM. This first step provided an overview of what kind of information is available on *in vitro* methodologies in the dossiers and how detailed the data are. The data were extracted from the dossiers and arranged in an Excel file.
 - In the second step, a thorough assessment of all the collected information was done. Data were analysed for each assay separately. Analysis of each TG-based assay was conducted and where available, more recent data from the literature was also evaluated.

2.1. Information extracted from dossiers

6. The dossiers containing information related to eleven MNMs (zinc oxide (ZnO), silicon dioxide (SiO₂), cerium dioxide (CeO₂), single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), gold (Au), silver (Ag), fullerene (C₆₀), dendrimers, nanoclay, and titanium dioxide (TiO₂)) tested under the Testing Programme (available at <http://www.oecd.org/chemicalsafety/nanosafety/>) were screened for applicability of *in vitro* methods to determine potential human hazard. The information was organized under the following subheadings in Excel files.

- Nanomaterial type
 - Physicochemical characterisation under *in vitro* conditions (e.g. using physiologically relevant fluids)
 - Biological endpoint addressed (e.g. genotoxicity)
 - *In vitro* method applied (e.g. MTT assay)
 - Test Guidelines followed (e.g. OECD TG 431 *In vitro* skin corrosion)
 - Good Laboratory Practice (GLP) study (yes/no)
 - Nanomaterial dispersion (type of dispersant, methods applied)
 - Controls used in the assay
 - Cell system used
 - Exposure details (e.g. duration of exposure)
 - Concentrations/doses applied
 - Conclusions from the assay (as reported in the dossier)
 - Additional observations
7. **Table 1** shows the OECD TGs used to assess *in vitro* toxicity endpoints and **Table 2** shows the non-TG methods applied under the Testing Programme for different MNMs.

Table 1. OECD TGs used to assess toxicity endpoints in *in vitro* systems under the Testing Programme

		ZnO	MWCNTs	Au	Fullerenes	SWCNTs	SiO ₂	CeO ₂	Ag	TiO ₂
<i>OECD TGs</i>										
Dermal absorption	OECD 428	y								
Skin corrosion	OECD 431	y	y							
Eye irritation	OECD 437	y								
Genotoxicity	OECD 471	y	y		y	y	y		y	
	OECD 473	y	y	y	y	y	y		y	
	OECD 476	y	y				y			y
	OECD 482						y			
	OECD 487						y			y

Note: No data was available for dendrimers and nanoclay.

OECD Test Guidelines can be download from:

<http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm>

Table 2. Cytotoxicity and genotoxicity assays used in the Testing Programme

Assay	ZnO	MWCNTs	Au	Dendrimers	SWCNTs	Fullerene	SiO ₂	CeO ₂	TiO ₂	
Cytotoxicity										
ATP CellTiter Glo			y							
Neutral red uptake	y			y					y	
LDH release		y	y	y	y	y	y	y	y	
MTT				y	y	y	y	y	y	
XTT			y							
Cell impedance			y						y	
Trypan Blue					y					
BrDU					y					
Alamar Blue					y					
WST-1					y		y	y		
Live/dead cell counting					y				y	
Colony forming efficiency		y						y		
Genotoxicity										
Comet assay					y		y		y	
DNA double-strands breaks					y					

Note: No data was available for Ag and nanoclay.

3. EVALUATION OF THE INFORMATION FROM TEST GUIDELINE (TG) AND NON-TG STUDIES EXTRACTED FROM THE DOSSIERS AND CURRENT LITERATURE

8. Each of the *in vitro* assays conducted based on existing OECD TGs was reviewed and evaluated in light of its applicability to testing MNMs considering whether or not adaptations were required to accommodate MNM properties. Since the Testing Programme ended in 2013, literature was reviewed to see if the TGs have been applied to test MNMs in other recent studies. The TGs are reviewed in the following sections and the information is organized under the following headings:

- Test guideline
 - General introduction
 - Applicability
 - Assay principle
 - General procedure
- Studies from the OECD Testing Programme where a TG was used to test MNMs
- Studies from current peer-reviewed literature
 - Study referenced
 - MNMs tested
 - Assay procedure
 - Results
- Overall summary and discussion

3.1. Toxicokinetics

3.1.1. TG 428: Skin Absorption: In Vitro Method

(OECD, 2004_[3])

General introduction

9. This test method (OECD, 2004c, adopted April 13, 2004) provide information on absorption of a test substance, applied to the surface of a skin sample separating the two chambers (a donor chamber and a receptor chamber) of a glass diffusion cell. Static and flow-through diffusion chamber are both acceptable. The details on conduct of the method are provided in OECD Guidance Document 28.

10. Skin samples of a specific thickness from human or animal sources can be used. Viable skin is preferred but, since it is not easily available, standardized non-viable skin can also be used provided that the integrity has been shown and documented prior to use.

11. Many inter-laboratory assessments, pre-validation studies, and validation studies on the same formulations *in vitro* and *in vivo* have been performed following this TG. All have demonstrated the utility of the *in vitro* approach in predicting *in vivo* absorption. EU

authorities have accepted the data from *in vitro* methods described by OECD TG 428 for many years.

12. Since MNMs are widely used in cosmetic products, their ability to penetrate the skin barrier is a relevant area of interest. Moreover, *in vitro* methods of dermal penetration are important for dermal delivery investigation of substances of pharmaceutical interest.

Applicability

13. Although not applicable for all situations and classes of chemicals, regulatory authorities in the EU now extensively use the *in vitro* method based on human skin as a stand-alone method as part of the approval process for the registration of new pesticide products. Recently, some of limitations of this *in vitro* method have been reviewed by ECHA (ECHA, 2016_[4]). Additional guidance for industry-specific sectors is also available (SCCS (Scientific Committee on Consumer Safety), 2010_[5]; OECD, 2011_[6]; EFSA, 2012_[7]).

Assay principle

14. The test substance, which ideally should be radiolabelled or fluorescent, is applied to the surface of a skin sample, derived from human or other animal species as pig and rat, separating the two chambers of a diffusion cell. Time of exposure should simulate potential human exposure (for example for industrial chemicals the exposure period should reflect occupational exposure). The receptor fluid is sampled at different time points (normally 24h to obtain an adequate absorption profile) and analysed for the presence of test chemical and/or metabolites. Crucial aspects are also the description of the skin origin, the evaluation of skin integrity before the use and the selection of an appropriate receptor fluid.

General procedure

15. Normally more than one concentration of the test substance is used in typical formulations, spanning the realistic range of potential human exposures. The application should mimic human exposure, normally 1 mg/cm² to 5 mg/cm² of skin for a solid and up to 10 µL/cm² for liquids is sufficient. The temperature must be kept constant (close to normal skin temperature 32 °C ± 1 °C) because passive diffusion of chemicals is affected by temperature. The receptor fluid, which must have an adequate capacity to solubilize the test substance and should not affect skin preparation integrity, is maintained in contact with the underside of the skin from the time of application until the end of its collection. For water soluble compounds receptor fluids are usually saline solutions, pH 7.4. For lipophilic test substances they can contain organic solvents.

Studies from the Testing Programme where TG 428 was used to test MNMs

Studies on ZnO MNMs - One study was reported

Assay procedure

16. In an *in vitro* dermal absorption study according to OECD TG 428 under GLP conditions, dermatomed pig skin mounted on static Franz-type diffusion cells was treated with nominal doses of 4 mg/cm² of a 10% oil/water formulation of Z-COTE - ZnO nanoparticles (NPs) coated with triethoxycaprylsilane [ZnO emulsion 10%, BASF AG],

corresponding to approximately 400 µg/cm² ZnO or 320 µg/cm² Zn²⁺) for 24 h. A semi-occlusive coverage was used. Porcine skin was used as diffusion barrier between the donor compartment of a diffusion cell and the receptor compartment, filled with the receptor medium (physiological saline solution containing 5% bovine serum albumin at test temperature of 31 °C to 33 °C). Skin preparations without treatment were used as control. Membrane integrity was checked by measuring its electrical resistance. Samples of receptor fluid (about 0.4 g/sample) were taken at various time intervals (3 h, 6 h, 12 h, and 24 h) after application of the test formulation to the skin and retained for analysis. No reference substance(s) were considered in this study.

17. At the end of exposure period, the test substance was removed from skin preparations by tape stripping and was also recovered from all other relevant compartment of each diffusion cell (considered as non-absorbed). Fractions present in the remaining skin after tape stripping and receptor chamber fraction are considered as recovery. Zn analyses were carried out by using Flame Atomic Absorption Spectrometry.

Results

18. The results indicated that no detectable amounts of Zn ions (Zn²⁺) from the Z-COTE containing formulation penetrated into or through the pig skin under the conditions reported in the study. Transmission electron microscopy (TEM) analysis revealed no difference in Zn content when comparing untreated skin preparations or those treated with either vehicle or test substance. No increased Zn concentrations were observed in the receptor fluid of substance treated skin as compared to vehicle treated. The mean total recoveries of Zn measured in diffusion cells equipped with 3 test skin samples were in the range of 102% to 107% and thus fulfil the quality criteria of OECD TG 428.

Non-TG studies under the Testing Programme

Studies on ZnO MNMs – Two studies were reported

○ *Study 1*

Assay procedure

19. Study (non-GLP) to determine whether porcine skin damaged by moderate ultraviolet B radiation (UVB) enhanced the penetration of 5% Z-COTE HP1 or 5% Z-COTE present in sunscreen oil/water formulation after exposure (BASF hydrophobic sunscreen formulations: CM 643, CM 644). The penetration of the nanoparticles (NPs) was investigated *in vitro* through dermatomed porcine skin (area of exposure 0.64 cm²) in flow-through diffusion cells, at 37 °C, 24 h after *in vivo* UVB exposure. The experiment was carried out with an occlusive coverage with perfusion system. Untreated skin of the same animal was used as negative control. The composition of the receptor fluid was the follow: 1.2mM KH₂PO₄, 32.7mM NaHCO₃, 2.5mM CaCl₂, 4.8mM KCl, 1.2mM MgSO₄*7H₂O, 118mM NaCl, 1200mg/L D-glucose, 4.5% BSA, 5 U/ml heparin, 30µg/ml amikacin, and 12.5U/ml penicillin G; pH 7.3-7.5, no data are reported about the solubility of the test substances in the receptor fluid. No reference substances were tested. The perfusate was collected every 2 h for the first 12 h, then every 4 h up to 24 h. Analysis was performed by applying light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as well as time-of-flight secondary ion mass spectrometry (TOF-SIMS) and inductively coupled plasma mass spectrometry (ICP-MS). Additionally, dermatomed skin samples of *in vivo* treated pigs were analysed.

Results

20. UVB-damaged skin did not enhance the penetration of the NPs in the provided sunscreen formulations. The NPs remained on the surface and within upper stratum corneum layers. In UVB unexposed skin, Z-COTE HP1 and Z-COTE remained on the surface as confirmed by EDS. TOF-SIMS analysis indicated that Zn penetrated into the epidermis under both treatment conditions. Nevertheless, no transdermal absorption was detected for ZnO nanoparticles in sunscreen formulations. In the *in vivo* study on dermatomed porcine skin ran in parallel showed that ZnO nanoparticles remained on the surface and upper stratum corneum layers of UVB exposed and unexposed skin. TEM analysis indicated that Zn penetrated into the stratum corneum while TOF-SIMS analysis indicated that it penetrated into the epidermis. Nevertheless, no transdermal absorption was detected.

○ Study 2

Assay procedure

21. Human epidermal skin penetration of a novel, transparent, nanoparticulate ZnO sunscreen formulation (ZnO dispersion of 60% siliconate coated ZnO and oil-in-water (O/W) emulsion sunscreen with 20% ZnO siliconate coated ZnO in caprylic capric triglyceride, respectively) was determined using Franz-type diffusion cells under non-GLP/guideline conditions. The membranes were mounted in static, horizontal Franz-type diffusion cells with an exposed surface area of approximately 1.3 cm². Treatment with the different formulations involved application of 10 µL/cm² for 24h of exposure. Information about receptor fluid composition, eventual reference substances tested, presence of negative controls and type of coverage were not reported in the dossier. Samples were collected at 12 h and 24 h intervals. The receptor fluid was analysed for the presence of zinc by ICP-MS and transmission electron microscopy (TEM) was used to verify the location of NPs in exposed membranes.

Results

22. The study reported that the epidermal penetration of Zn was negligible following topical application of the nanoparticulate formulation to human epidermis *in vitro*. Less than 0.03% of the applied Zn content penetrated the epidermis. No particles could be detected in the lower stratum corneum or viable epidermis by EM, suggesting that minimal NP penetration occurs through the human epidermis.

Studies from current literature

Studies on TiO₂ MNMs – Two studies have been reported

○ Study 1

Study referenced

23. Senzui M, Tamura T, Miura K, Ikarashi Y, Watanabe Y, Fujii M. Study on penetration of titanium dioxide (TiO₂) nanoparticles into intact and damaged skin *in vitro*. J Toxicol Sci 2010, 35: 107–113. 10.2131/jts.35.107 (Senzui et al., 2010_[8])

MNMs tested

24. Four different types of rutile TiO₂ were tested: T-35, 35 nm, uncoated; TC-35, 35 nm, with alumina/silica/silicone coating; T-disp, 10x100 nm, mixture of alumina coated and silicon coated particles, dispersed in cyclopentasiloxane; T-250, 250 nm, uncoated.

Assay procedure

25. Skin penetration of the TiO₂ NPs was determined with *in vitro* intact, stripped, and hair-removed skin of Yucatan micropigs. 2 µL of TiO₂ suspension (10% in cyclopentasiloxane) was applied to the area of skin (approximately 1 cm²). The skin was placed on a modified Franz-type diffusion cell. Silicone was used as control. After 24 h, the receptor fluid (pH 7.1 isotonic phosphate buffer solution) was collected and Ti concentration in skin was determined by ICP-MS. Application amount and period were in accordance with the standard SPF Test Method of the Japan Cosmetic Industry Association (1999) and OECD TG 428 (2004) as reported in the publication.

Results

26. ICP-MS results indicate that TiO₂ NPs did not penetrate into viable skin. However, after hair removal, some particles penetrated relatively deeply into the skin, apparently through hair follicles. Indeed, SEM and TEM observation showed that Ti penetrated into vacant hair follicles when it was applied on skin from which hair was removed.

○ Study 2

Study referenced

27. Crosera, M.; Prodi, A.; Mauro, M.; Pelin, M.; Florio, C.; Bellomo, F.; Adami, G.; Apostoli, P.; De Palma, G.; Bovenzi, M.; Campanini, M.; Filon, F.L. Titanium dioxide nanoparticle penetration into the skin and effects on HaCaT cells. Amorim M, ed. International Journal of Environmental Research and Public Health. 2015;12(8):9282-9297. doi:10.3390/ijerph120809282 (Crosera et al., 2015_[9])

MNMs tested

28. Commercially available TiO₂ nanopowder (CAS 13463-67-7, n. 677469 provided by Sigma Aldrich—Milan, Italy).

Assay procedure

29. TiO₂ NP suspensions (concentration 1.0 g/L or 606 µg/cm²) in synthetic sweat solution were applied on static Franz cells for 24 h using intact and needle-abraded human skin. TiO₂ content into skin and receiving phases was determined by TEM analysis and ICP-Atomic Emission Spectroscopy (ICP-AES). Skin integrity was tested before and after each experiment using electrical conductivity. The receptor compartment had a mean volume of 14.0 mL and was maintained at 32 °C and the receptor fluid was a physiological solution (final pH 7.35). The mean exposed skin area was 3.29 cm² and the average membrane thickness was 1 mm. Synthetic sweat was used as negative control. At the end of the experiment, skin pieces were digested and analysed to determine metal concentration by ICP-AES.

Results

30. No TiO₂ permeation was observed after 24 h of exposure of the skin to TiO₂ NPs both in intact and in damaged skin. TiO₂ was found in the epidermal layer after 24 h of exposure (0.47 ± 0.33 µg/cm²; uncertainty values for this study are standard deviation

values of six samples) while in the dermal layer, the concentration was below the limit of detection. Damaged skin, in its whole, has shown a similar concentration ($0.53 \pm 0.26 \mu\text{g}/\text{cm}^2$). The study reported that TiO_2 NPs cannot permeate intact and damaged skin and can be found only in the stratum corneum and epidermis.

Studies on TiO_2 and ZnO MNMs – One study has been reported

Study referenced

31. Monteiro-Riviere NA, Wiench K, Landsiedel R, Schulte S, Inman AO, Riviere JE. Safety evaluation of sunscreen formulations containing titanium dioxide and zinc oxide nanoparticles in UVB sunburned skin: An *in vitro* and *in vivo* study. *Toxicological Sciences*. 2011; 123(1):264–280. doi:10.1093/toxsci/kfr148 (Monteiro-Riviere et al., 2011_[10])

MNMs tested

32. Four hydrophobic and hydrophilic sunscreen formulations containing commercially available TiO_2 and ZnO products (BASF, Ludwigshafen, Germany) were used in the study: CM 630 and CM 634 (10% TiO_2 in w/o formulation, rutile 14–16 nm, specific surface area $100 \text{ m}^2/\text{g}$), CM 643 (5% ZnO (Z-COTE HP1)) in o/w formulation), and CM 644 (5% ZnO (Z-COTE) in o/w formulation), both with a mean size of 140 nm and a specific surface area of $12 \text{ m}^2/\text{g}$ to $24 \text{ m}^2/\text{g}$.

Assay procedure

33. Porcine skin was mounted in flow-through diffusion cells maintained at 37°C with a dosing area of 0.64 cm^2 and equilibrated in perfusate (saline solution added with 1200 mg/L D-glucose, 4.5% bovine serum albumin, 5 U/mL heparin at pH 7.4) at a flow rate of 2 mL/h for 30 minutes prior to dosing. Upon completion of dosing, perfusion was resumed, and the perfusate was collected every 2 h for the first 12 h, then every 4 h up to 24 h. Perfusate samples from each timed collection were capped and stored at 4°C for TiO_2 and ZnO analysis by ICP-MS. Ti and Zn content in porcine skin samples was determined by time of flight-secondary mass spectrometry (TOF-SIMS).

Results

34. TOF-SIMS showed TiO_2 within epidermis and superficial dermis, whereas ZnO was limited to stratum corneum and upper epidermis in both normal and damaged skin treated with all sunscreen formulations. UVB-sunburned skin slightly enhanced penetration of TiO_2 or ZnO NPs present in sunscreen formulations. In most cases, TiO_2 penetration into the stratum corneum was greater than ZnO. No evidence of systemic absorption was found since NPs were not seen in the perfusate.

Studies on SiO_2 MNMs - One study has been reported

Study referenced

35. Staroňová, K., Nielsen, J. B., Roursgaard, M. and Knudsen, L. E. Transport of SiO_2 nanoparticles through human skin. *Basic Clin Pharmacol Toxicol*. 2012;111: 142–144. doi:10.1111/j.1742-7843.2012.00873.x (Staroňová et al., 2012_[11])

MNMs tested

36. Silica NPs 25 nm, (Fluorescent SiO_2 Nanospheres, fluorescence of Rhodamine; Microspheres-Nanospheres, Cold Spring, NY, USA).

Assay procedure

37. Transport experiments were performed *in vitro* on Franz diffusion cells according to OECD TG 428. Human skin samples originated from female donors who had undergone breast surgery. Skin samples were prepared to a thickness of 900 μm . The receptor chambers were filled with a medium composed of 0.9% sodium chloride (NaCl) supplemented with gentamycin (900 $\mu\text{L/L}$). 106 μL of the SiO_2 NPs in a concentration of 1000 $\mu\text{g/mL}$ test substance was dosed on the epidermal side of the skin, with the diffusion area of 2.12 cm^2 (50 $\mu\text{L/cm}^2$). Two unexposed skin samples were used as control. Samples of receptor fluid were taken from the stirred receptor fluid after 4 h, 6 h, 8 h, 12 h, and 24 h. At the end of the experiment, non-absorbed particles from the epidermal surface were removed by washing with medium. The fluorescence of the SiO_2 NPs in the receptor fluid and in the donor wash samples was measured by the Fluoroskan Ascent device. Presence of NPs in the different layer of the skin was determined at the end of the transport experiments by confocal microscopy.

Results

38. SiO_2 NPs were primarily identified in the epidermis with minor deposition in the upper dermis and with an intensity diminishing with increasing depth. No permeation through the skin was reported.

Studies on Au MNMs – Two studies have been reported

o Study 1

Study referenced

39. Filon FL, Crosera M, Adami G, Bovenzi M, Rossi F, Maina G. Human skin penetration of gold nanoparticles through intact and damaged skin. *Nanotoxicology*. 2011;5(4):493-501. doi: 10.3109/17435390.2010.551428 (Larese Filon et al., 2011_[12])

MNMs tested

40. Au NPs (diameter of 12.6 nm \pm 0.9 nm.) were synthesized by reducing tetrachloroauric acid with sodium citrate and characterized using TEM.

Assay procedure

41. Experiments were performed using the Franz diffusion cell method with intact and damaged human skin at 32 °C. A physiological solution (final pH = 7.35) was used as the receptor fluid. The exposure chambers of Franz diffusion cells were filled with the solution (0.5 mL or 1.5 mL) containing 100 mg/L of Au NPs (15 mg/cm^2 or 45 mg/cm^2) and diluted 1:3 with synthetic sweat (pH 4.5) or milliQ water for the 4 different exposure scenarios.

Results

42. Skin absorption was reported to be dose dependent. Mean Au content of 214.0 \pm 43.7 ng/cm^2 (uncertainty values for this study are standard deviation values) and 187.7 \pm 50.2 ng/cm^2 were found in the receiving solutions of cells where the Au NPs solution was applied in higher concentration on intact skin and on damaged skin, respectively. 24 h Au flux permeation was 7.8 \pm 2.0 $\text{ng/cm}^2 \text{ h}$ and 7.1 \pm 2.5 $\text{ng/cm}^2 \text{ h}$ in intact and damaged skin, respectively, with a lag time less than 1 h. TEM analysis on skin samples and chemical analysis using ICP-MS showed the presence of Au NPs into epidermis and dermis. The study reported that Au NPs can permeate the skin in greater amount in

comparison to other NPs, such as Ag NPs in an *in vitro* diffusion system. Moreover, since Au NPs do not release Au ions in physiological condition, this penetration happens only for NPs.

○ **Study 2**

Study referenced

43. Lee O, Jeong SH, Shin WU, Lee G, Oh C, Son SW. Influence of surface charge of gold nanorods on skin penetration. *Skin Res Technol.* 2013;19(1):e390-6. doi: 10.1111/j.1600-0846.2012.00656.x (Lee et al., 2013_[13])

MNMs tested

44. Au nanorods with different surface charge were synthesized using the cetyltrimethyl ammonium bromide (CTAB) method and sequentially coated with poly(sodium-4-styrenesulfonate (PSS) and poly(diallyldimethylammonium chloride) (PDADMAC). The diameter and length of the nanorods was assessed using TEM and was measured to be 18 X 40 nm.

Assay procedure

45. To evaluate the influence of the surface charge of Au nanorods (GNs) on diffusion through the skin, *in vitro* permeation studies using hairless mouse skin in Franz-type diffusion cells were carried out. Receptor chambers were filled with 4 mL PBS, and GNs (100 µL, 500 µg/mL) were applied to the donor chambers. Samples of receptor fluid were taken at various time intervals (8 h, 24 h, and 48 h). The concentration of permeant particles was investigated by ICP-MS and NPs penetration in skin samples was visualized by TEM.

Results

46. The study reported that negatively-charged GNs penetrate skin better and more rapidly than positively charged GNs. Consistent with the TEM observations, higher concentrations of negatively-charged particles were detected in samples of receptor fluid 48 h after exposure ($P < 0.01$). No significant differences were observed at 8 h and 24 h after exposure.

Studies on Ag MNMs - One study has been reported

Study referenced

47. Bianco C, Adami G, Crosera M, Larese F, Casarin S, Castagnoli C, Stella M, Maina G. Silver percutaneous absorption after exposure to silver nanoparticles: A comparison study of three human skin graft samples used for clinical applications. *Burns : journal of the International Society for Burn Injuries.* Nov 2014; 40; (2):1390-1396, doi: 10.1016/j.burns.2014.02.003 (Bianco et al., 2014_[14])

MNMs tested

48. Polymer-coated Ag NPs (content of Ag: 25% mass fraction, polyvinylpyrrolidone: 75%) were supplied by NanoAmor Materials Inc. (Houston, TX, USA).

Assay procedure

49. Study was aimed to evaluate Ag NP penetration using fresh, cryopreserved, and glycerolized human skin grafts after exposure to a suspension of Ag NPs to evaluate the use of cryopreserved human skin for *in vitro* experiments (according to TG 428) to predict *in vivo* penetration. A static Franz diffusion cell apparatus were used. The receptor fluid was filled with 14.0 mL of physiological solution maintained at 32°C. The mean of the exposed area was 3.29 cm² and the average of the membrane thickness was 0.7 mm. Two untreated skin grafts were used as blank for each experiment. At selected intervals (2 h, 4 h, 8 h, 12 h, 21 h, and 24 h), 1.5 mL of the dermal bathing solution was collected for subsequent analyses and immediately replaced with an equal volume of fresh physiological solution. After 24 h, the dermal bathing solution and the donor phase of each diffusion cell were recovered for the analysis. Skin grafts were also removed and digested to determine Ag concentration by ICP-MS.

Results

50. The study reported that Ag can pass the skin barrier reaching the dermis. Permeation through glycerolized skin is significantly higher compared to both fresh and cryopreserved skin. No significant differences were found between percutaneous absorption of Ag in cryopreserved and fresh skin confirming the use of cryopreserved skin in *in vitro* experiments as a good model for skin permeation evaluation.

Overall summary and discussion

- *In vitro* skin absorption studies reported in the OECD WPMN Testing Programme were only conducted with ZnO NPs. Not all the studies reported in the dossier have been carried out according to TG 428.
- Several *in vitro* skin absorption studies were reported in recent literature. The studies have been performed to test different MNMs and several commercial formulations containing MNMs.
- Among the NPs tested, permeation was found for Au and Ag NPs but was not detected within the observation period for TiO₂, ZnO, and SiO₂ NPs.
- As deeply discussed in the OECD Expert Meeting on Toxicokinetics of Manufactured Nanomaterials (OECD, 2016_[15]), TG 428 should be adapted for testing on MNMs, since there are many critical points in the protocol that may not be adequate for MNMs. In particular, the duration of the observation time, the sampling time, the influence of mechanical process in particles translocation, the MNMs solubility and the compatibility of the receptor fluid with MNMs need to be further explored.

3.1.2. TG 431: In Vitro Skin Corrosion: Reconstructed Human Epidermis (RHE) Test Method

(OECD, 2016_[16])

General introduction:

51. The OECD TG on using the *in vitro* methods to assess skin corrosion (TG 431) allows identification of non-corrosive and corrosive substances and mixtures and partial sub-categorization of corrosives in accordance to the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Four validated test methods EpiSkin™, EpiDerm™, SkinEthic™, and epiCS® (that use reconstructed human epidermis), are included in the TG.

Applicability

52. In absence of the scientific evidence demonstrating otherwise, TG 431 is applicable to chemicals and mixtures but does not allow testing of gases and aerosols.

Assay principle

53. TG 431 is based on the principle that corrosive chemicals are able to penetrate the stratum corneum and are toxic to the underlying layers.

General procedure

54. At least two tissue replicates are used for each test chemical in addition to positive and negative controls for each exposure time. A minimum dose of 70 $\mu\text{L}/\text{cm}^2$ or 30 mg/cm^2 should be used. Depending on the type of tissue used, two or three exposure times are recommended.

Studies from the Testing Programme where TG 431 was used to test MNMs

Studies on MWCNTs - Two studies were reported

Assay procedure

55. The studies tested two different types of MWCNTs (Baytubes and Nanocyl 7000). No details of the studies were included, so these are not further discussed here.

Studies on ZnO MNMs - Two studies were reported

Assay procedure

56. The studies tested Z-COTE HP1 which are ZnO NPs coated with triethoxycaprylylsilane. Both the studies were GLP guideline studies and followed TG 431. For both studies two EpiDermTM tissues each were treated with the test item for 3 minutes and 1 h, respectively. Water was used as negative control and 8 N KOH as positive control. At the end of the exposure period the cell viabilities of the treated tissues were measured using MTT test and calculated as percent relative to the negative control.

Results

57. Based on the results of the *in vitro* studies reported under the Testing Programme, Z-COTE HP1 was classified as non-corrosive to skin while the positive control was classified as corrosive to skin as expected.

Studies from current literature

Study referenced

58. Choi J, Kim H, Choi J, Oh SM, Park J, Park K. Skin corrosion and irritation test of sunscreen nanoparticles using reconstructed 3D human skin model. *Environmental Health and Toxicology*. 2014; 29: e2014004. doi:10.5620/eht.2014.29.e2014004 (Choi et al., 2014_[17])

MNMs tested

59. Choi et al. tested two different types of MNMs (ZnO and TiO₂) and their mixture (ZnO plus TiO₂) used in the sunscreens. ZnO (< 35nm) were coated with 3-aminopropyl triethoxysilane. TiO₂ NPs (P25, 21 nm-size, ca. 5 hydroxyl-groups/nm², Aeroxide®, Evonik) used in this study were also included in the OECD Testing Programme. The test MNMs and their mixtures were suspended in deionized water for characterization and cell exposures.

Assay procedure

60. Three replicates of KeraSkin™ tissue models were exposed to ZnO and TiO₂ NPs (ZNPs and TNPs), and their mixture for 3 minutes or 1 h. The concentration of MNMs was 25% in deionized water and 30 µL suspension volume was added to the skin model. In the mixture, one volume of 50% ZNPs and one volume of 50% TNPs were mixed to finally produce 25% of MNMs and total 30 µL suspension volume was applied to the skin model. 8N KOH was used as positive chemical and eugenol was used as negative chemical. The results were compared to *in vivo* data using rabbits (Lee et al., 2013_[13]).

Results

61. All the test materials ZNPs, TNPs and their mixture used in this study were found to be non-corrosive in *in vitro* and *in vivo* studies.

Overall summary and discussion

62. These studies demonstrate the probable applicability of TG 431 to assess the skin corrosion potential of MNMs based on the following points:

- Based on TG 431 the tissue models are washed before the cell viability is assessed reducing the chance of MNM interference with the MTT assay.
- The studies applied TG 431 to test mixtures of MNMs.

Similarities were reported between the outcomes of *in vitro* conducted using TG 431 and *in vivo* study (Choi et al., 2014_[17]). However, as in the case of TG 428 some critical issues related to the protocol are identified (e.g. the lack of the circulation in the subcutis, the relevant length of exposure, surface area of exposure, compatibility of the receptor fluid for MNMs), suggesting that the amendment of this guideline might be needed in view of MNMs testing application.

3.2. Eye irritation and damage

3.2.1. TG 437: Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

(OECD, 2009_[18])

General introduction

63. The OECD TG on using the Bovine Corneal Opacity and Permeability (BCOP) *in vitro* test method to assess impacts of chemicals (substances and mixtures) on the eye (TG 437) can be used to identify chemicals inducing serious eye damage (GHS 1) and for chemicals that do not require classification for eye irritation or serious eye damage under

the UN GHS classification system. The BCOP test method is not recommended for the identification of test chemicals that should be classified as irritating or mildly irritating to eyes (see UN GHS Category classifications).

Applicability

64. In absence of the scientific evidence demonstrating otherwise, TG 437 is applicable to chemicals and mixtures but there was a high false positive rate for alcohols and ketones and a high false negative rate for solids in the validation database. The BCOP test method is an *in vitro* test method that can be used under certain circumstances and with specific limitations for eye hazard classification and labelling of chemicals. While it is not considered valid as a stand-alone replacement for the *in vivo* rabbit eye test, the BCOP test method is recommended as an initial step within a testing strategy such as the Top-Down approach to identify chemicals inducing serious eye damage, i.e. chemicals to be classified as UN GHS Category 1, without further testing. The BCOP test method is also recommended to identify chemicals that do not require classification for eye irritation or serious eye damage, as defined by the UN GHS (UN GHS No Category) within a testing strategy such as the Bottom-up approach. However, a chemical that is not predicted as causing serious eye damage or as not classified for eye irritation/serious eye damage with the BCOP test method would require additional testing (*in vitro* and/or *in vivo*) to establish a definitive classification.

Assay principle

65. The BCOP assay is an organotypic model utilizing bovine cornea that can be used to provide a quantitative measurement of changes in corneal capacity and permeability with an opacitometer and a visible light spectrophotometer, respectively, after application of the chemical. This assay uses the isolated corneas from the eyes of freshly slaughtered cattle.

General procedure

66. Bovine corneas, confirmed to be free of defects, are dissected and mounted in special holders and equilibrated in the test chamber for at least one hour. Liquids or surfactants are added undiluted to the eyes, or solids are tested by direct application onto the corneal surface using the open chamber method. After the exposure period, the test chemical is removed by washing and the opacity and permeability of each cornea are recorded. Concurrent negative or solvent/vehicle controls and positive controls are included with each experiment.

Studies from the Testing Programme where TG 437 was used to test MNMs

Studies on ZnO MNMs - One study was reported

Assay procedure

67. The study tested NM-110 Zinc Oxide. The capacity for the NM-110 to cause serious damage to the eyes was tested using the BCOP test. 750 µL of the 20% mass/volume test substance preparation in deionized water was applied for 4 h using the open chamber method. Imidazole in de-ionized water was used as the positive control.

Results

68. The results of this study showed a lack of serious eye damage. The mean IVIS score was 49.5 and 16.1 for two runs of the test*. Negative and positive control values are also reported. Histological analysis revealed changes indicating minimal eye irritation.

*Note

69. There is a discrepancy between the classification suggested in the dossier (no classification on page 223 of ZnO NM dossier) and that in the 2013 version of TG 437. Based on the table on page 11 of TG 437, no prediction can be made for IVIS scores between 3 and 55 and additional assays are needed but within the ZnO NM dossier it states that an IVIS score ≤ 55 predicts no risk of serious damage to the eyes.

Studies from current literature

Study referenced

70. Kolle, S. N., Sauer, U. G., Moreno, M. C., Teubner, W., Wohlleben, W., & Landsiedel, R. Eye irritation testing of MNMs using the EpiOcular eye irritation test and the bovine corneal opacity and permeability assay. Part Fibre Toxicol. (2016).13, 18. doi: 10.1186/s12989-016-0128-6 (Kolle et al., 2015_[19])

MNMs tested

71. This study tested 16 OECD representative MNMs¹ comprising of six different types of TiO₂ (anatase (NM-100, NM-101, NM-102), rutile (NM-103, NM-104), and rutile-anatase (NM-105)), ZnO (uncoated (NM-110) and coated (NM-111)), amorphous SiO₂ (NM-200 and NM-203), uncoated CeO₂ (NM-211 and NM-212), Ag (NM-300 K), and three MWCNTs of different lengths and diameters (NM-400, NM-401, and NM-402). For the EpiOcularTM-EIT, the test materials were applied undiluted and for BCOP assay, the MNMs were suspended in highly deionized water to achieve final concentrations of 20% (mass/volume) suspension.

Assay procedure

72. For the EpiOcularTM-eye irritation test (EIT), 2 tissues were used for each treatment group (test material, negative control, and positive control). The dry-powder or liquid test items, were applied to cover the entire tissue surface. 50 μ L highly deionized water was used as a negative control and methyl acetate was used as a positive control. After test material application, the tissues were incubated for 90 min (solids; variant 1), 6 h (solids; variant 2), or 30 min (liquids). The tissues were then washed and incubated at standard culture conditions for 18 h (solids; variants 1 and 2) or 2 h (liquids). After post-exposure incubation, the tissues were tested for viability using MTT assay.

73. For the BCOP assay, three corneas were used for each treatment group (test material, negative control, and positive control). 750 μ L of deionized water was used as a negative control and 20% (weight/volume) imidazole solution was used as a positive control. To prepare the suspensions, the MNM powders were added to de-ionized water at a concentration of 20 % (mass/volume) and stirred with a magnetic mixer. The TiO₂ (NM-103 and NM-104), ZnO (NM-110) and SiO₂ (NM-200) powders were dispersed with a high-speed homogenizer. 750 μ L of the diluted (20% (weight/volume), for TiO₂,

¹ See the JRC Nanomaterials Repository of representative industrial nanomaterials <https://ec.europa.eu/jrc/en/scientific-tool/jrc-nanomaterials-repository>

CeO₂, SiO₂, and ZnO NM-110) or undiluted (Ag NM-300 K and Ag NM-300 K DIS [i.e. the dispersant without silver nanoparticles], and SiO₂), test material preparation was applied directly to the epithelial surface of the cornea. For ZnO (NM-111) and MWCNT (NM-401), 33 mg and 48 mg of undiluted dry-powder, respectively, were applied with a sharp spoon. 20% (mass/weight) dry suspensions of MWCNT NM-400 and NM-402 were prepared by mixing 750 mg MWCNT in highly de-ionized water shortly before application by stirring. Quartz dust DQ12, talc, and the three organic pigments (Pigment Red 57:1, Pigment Yellow 95, and Pigment Black 32) were applied undiluted (120 mg, 80 mg, 48 mg, 45 mg, and 40 mg). For dry-powder test materials and SiO₂-suspension, the corneas were incubated for 4 h as prescribed in the OECD TG. Ag NM-300 K, and Ag NM-300 K DIS were applied for 10 min followed by 2 h post incubation as prescribed for liquids. After incubation, the controls and the test material were removed and the respective epithelia were washed at least 3 times with Eagle's MEM (containing phenol red) and once with Eagle's MEM (without phenol red). Fresh Eagle's MEM (without phenol red) was added and the final corneal opacity and permeability were measured to calculate the *in vitro* irritancy score for all the tissues. Histopathological evaluation was also conducted.

Results

74. All dry-powder MNMs consistently showed lack of eye irritation potential in both the *in vitro* EpiOcular™-EIT and the BCOP assay. Of the three suspended test materials (2 of which being MNMs), SiO₂-suspension was non-irritating in both *in vitro* tests but Ag NM-300 K and Ag NM-300 K DIS showed inconclusive results in the BCOP assay.

Overall summary and discussion

75. These studies reported that TG 437 is generally applicable for assessment of eye irritation or serious eye damage potential of MNMs. However, application of TG 437 will require further evaluation of the method and amendment of the protocol to accommodate MNM related aspects such as thorough characterization of the MNMs (powders and/or suspensions). Results were generally in agreement between TG 437 and an EpiOcular™-EIT assay. It has to be noted however, that as concluded by the SCCP in the Guidance on the Safety Assessment of Nanomaterials in Cosmetics (SCCS (Scientific Committee on Consumer Safety), 2012_[20]) “no specific validation has been performed for nanomaterials, although there is no clear scientific basis against the use of the method for nanomaterials. It should, however, be kept in mind that nanomaterials can aggregate/agglomerate in the suspension or can absorb dispersant. These aspects should be verified. Some nanomaterials present in opacity measurements may affect the result, and these should be avoided to allow consistent interpretation of results. Both methods measure the leakage of fluorescein. Possible artifacts due to absorption of the dye to nanomaterials should be verified and eliminated”.

3.3. Genotoxicity

76. A number of assays (TG and non-TG) were used to assess the genotoxicity of MNMs under the Testing Programme (see table 3). These assays have been evaluated under this section.

Table 3. Overview of *in vitro* genotoxicity assays used in the OECD Testing Programme to test the various MNMs

MNM	TG 471	TG 473	TG 476	TG 487	Comet assay	Double stands breaks assay (H2AX phosphorylation)
Au NPs	x	x				
Ag NPs	x	x				
ZnO (NM-110) - microscale		x	x			
ZnO (NM-111)	x	x	x			
ZnO (NM-113)	x	x	x			
TiO ₂ (NM-101)					x	
TiO ₂ (NM-102)			x	x	x	
TiO ₂ (NM-103)			x	x	x	
TiO ₂ (NM-104)			x	x	x	
TiO ₂ (NM-105)	x		x	x	x	
SWCNTs (Nikkiso)	x	x				
SWCNTs (Super Growth)	x	x				
SWCNTs (CNI)					x	
SWCNTs (EliCarb)					x	
SWCNTs (Arc)						x
SWCNTs (Sigma Aldrich)					x	
SWCNTs (NIST)					x	x
SWCNT (Heji)					x	
SWCNT (COCC)					x	
Fullerenes	x	x				
SiO ₂ (NM-200)		x	x	x	x	
SiO ₂ (NM-201)			x	x	x	
SiO ₂ (NM-202)	x	x	x	x	x	
SiO ₂ (NM-203)	x	x	x	x	x	
SiO ₂ (NM-204)		x				
MWCNTs (Baytubes)	x	x				
MWCNTs(Graphistrength C100)	x	x	x			
MWCNTs (Mitsui -7)	x					

Source: <http://www.oecd.org/chemicalsafety/nanosafety/>
<http://www.oecd.org/chemicalsafety/nanosafety/dossiers-and-endpoints-testing-programme-manufactured-nanomaterials.htm>
<http://publications.jrc.ec.europa.eu/repository/handle/JRC64075>

3.3.1. TG 471: Bacterial Reverse Mutation Test

(OECD, 1997_[21])

General introduction

77. The bacterial reverse mutation test (also known as Ames test) uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. It is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests. There are examples of mutagenic agents which are not detected by this test; reasons for these shortcomings can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability. On the other hand, factors which enhance the sensitivity of the bacterial reverse mutation test can lead to an overestimation of mutagenic activity.

Applicability

78. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds. The bacterial reverse mutation test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds (e.g. certain antibiotics) and those which are thought (or known) to interfere specifically with the mammalian cell replication system (e.g. some topoisomerase inhibitors and some nucleoside analogues). In such cases, mammalian mutation tests may be more appropriate.

79. The TG 471 does not contain any MNM specific considerations. However, the applicability of this test for MNMs has been extensively discussed (see the report from the OECD Expert Meeting on Genotoxicity of Manufactured Nanomaterials [ENV/JM/MONO(2014)34], concluding that "The use of the Ames test (TG 471) is not a recommended test method for the investigation of the genotoxicity of nanomaterials. The test guidelines programme should consider modifying the applicability domain within this test guideline accordingly.") (OECD, 2014_[22]). Also the SCCS in the Guidance on Safety Assessments of Nanomaterials in Cosmetics (SCCS (Scientific Committee on Consumer Safety), 2012_[20]) concluded that "although reports can be found on positive bacterial reverse mutation test, there are doubts if the Ames test is an accurate representative test for genotoxicity" because bacterial cells lack uptake of nanomaterials through endocytosis, and some nanomaterials have bactericidal activity. Therefore this test has not been regarded suitable for testing nanomaterials.

Assay principle

80. The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by

their ability to grow in the absence of the amino acid required by the parent test strain. The bacterial reverse mutation test utilises prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted generally require the use of an exogenous source of metabolic activation to ensure that potential genotoxicity of liver metabolites of the tested compound is also detected. The most commonly used system is the S9 mixture, a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents (generally rats) treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbital and β -naphthoflavone.

81. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals. The SCCS also discussed the usefulness of using the metabolic activation system (SCCS (Scientific Committee on Consumer Safety), 2012_[20]) and concluded that although most insoluble nanomaterials (e.g. some metals) are not metabolised, but “proteins in the metabolic activation system may interfere with the nanomaterial and alter bioavailability of the nanomaterial, and thus reduce sensitivity of the assay. Notwithstanding this it should be verified whether some nanomaterials can be metabolised, e.g. organic nanomaterials, or some inorganic nanomaterials may become coated with organic substances, or surface modified with organic functional groups.”

General procedure

82. Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the pre-incubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after two or three days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

Studies from the Testing Programme where TG 471 was used to test MNMs

83. The TG 471 was used in the OECD WPMN Testing Programme to test Au NPs, Ag NPs, ZnO NPs, SiO₂ NPs, TiO₂ NPs, SWCNTs, MWCNTs, and fullerenes.

Studies on Au MNMs - One study was reported

Assay procedure

84. The Au NPs were tested in *S. typhimurium* TA97a, TA98, TA100 and TA102. The particles were dispersed in distilled water. A single dose exposure was performed at a concentration range of 10 μ g to 162 μ g Au NPs/plate was tested. No information on duration of exposure was provided. The following positive controls were used: sodium azide (5 μ g/plate) for TA100 strain; Mitomycin C (0.5 μ g/plate) for TA102; 2-nitrofluorene for TA98 (2.5 μ g/plate); and 9-aminoacridine (50 μ g/plate) for TA97a strain.

Deviation from the current TGs

85. The assay was performed without metabolic activation.

Results

86. No dose related increase in revertants was observed following treatment with Au NPs. Following incubation of the TA98 strain with the Au NPs, the bacteria were observed using dark-field microscopy and hyperspectral imaging using a Cytoviva instrument. Au NPs were thus regarded as not mutagenic under the test conditions; however, this may be due to the observation that the NPs did not enter the bacteria based on imaging data.

Studies on Ag MNMs - One study was reported

Assay procedure

87. The Ag NPs were tested in *S. typhimurium* TA 1535, TA 1537, TA 98, and TA 100, and *E. coli* WP2uvr A. The NPs were dispersed in 1% citrate solution. In *S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2uvr A, a dose range of 0 µg/plate, 1 µg/plate, 2 µg/plate, 4 µg/plate, 8 µg/plate, 16 µg/plate, 32 µg/plate, and 62 µg/plate was tested without S9 mixture and 0 µg/plate, 8 µg/plate, 16 µg/plate, 32 µg/plate, 62 µg/plate, 126 µg/plate, 250 µg/plate, and 500 µg/plate with S9 mixture. In *S. typhimurium* TA100 with S9 mixture 0 µg/plate, 4 µg/plate, 8 µg/plate, 16 µg/plate, 32 µg/plate, 62 µg/plate, 126 µg/plate, and 250 µg/plate were tested. No further details on the exposure are given.

88. The following positive controls were used: 1) without S9 mixture (Furylfuramide for TA98, TA100 and WP2uvrA, sodium azide for TA1535, and 9-aminoacridine hydrochloride hydrate for TA1537) and; 2) with S9 mixture: 2-aminoanthracene for all tested bacterial species.

Deviation from the current TGs

89. Details on test item, strain characteristics, media used, number of cells/culture, treatment procedure, incubation time and temperature, individual and summary tables of results and historical negative (solvent/vehicle) and positive control data not reported.

Results

90. Ag NPs did not induce an increase in bacterial colony formation, and thus were considered not mutagenic under the test conditions.

Studies on SiO₂ MNMs - Two studies were reported

Assay procedure

91. The studies tested two different types of SiO₂ MNMs (NM-202 and NM-203). Both MNMs were tested in *S. typhimurium* TA 1535, TA 1537, TA 98, and TA 100 at concentrations of 667 µg/plate, 1000 µg/plate, 3333 µg/plate, 6667 µg/plate, and 10.000 µg/plate, with and without metabolic activation (S9 mixture). No further details on the exposure are provided.

Results

92. In both studies SiO₂ MNMs did not induce an increase in bacterial colony formation, and thus were considered not mutagenic under the test conditions.

Studies on TiO₂ MNMs - One study was reported

Assay procedure

93. The material was dispersed in DMSO in concentration of 50 mg/mL with 10-minutes sonication and used for the testing after stepwise dilution. NM-105 were tested in *S. typhimurium* TA 1535, TA 1537, TA 98, and TA 100, at concentrations of 5000 µg/plate, 2500 µg/plate, 1250 µg/plate, 625 µg/plate, and 312.5 µg/plate, without metabolic activation. No further details on the exposure are provided.

Results

94. TiO₂ MNM-105 were considered not mutagenic at the tested doses. Precipitation of test substance was observed at 1250 µg/plate and higher doses.

Studies on ZnO MNMs - Two studies were reported.

Assay procedure

95. The studies tested uncoated non-nanosized zinc oxide (NM-113) and Z-Cote Max (based on NM-111), using the Bacterial Reverse Mutation Assay according to OECD 471 under GLP conditions. Standard plate and pre-incubation tests were conducted in the absence of S9 mixture (NM-113) or +/- S9 mixture (Z-COTE Max) with doses of 20 µg/plate, 100 µg/plate, 500 µg/plate, 2500 µg/plate, and 5000 µg/plate using the *S. typhimurium* strains TA 1535, TA 100, TA 102, TA 1537, and TA 98.

96. NM-113 particles were suspended in three different media: water, fetal bovine serum (FBS), and bovine lung surfactant (ALVEOFACT®). The test substance was weighed and topped up with the chosen vehicle to achieve the required concentration of the stock dispersion. Then, the dispersion was stirred in a closed vessel for 24 h at 1,200 rpm (126 rad/s) at room temperature. The concentrations were further diluted from the stock solution according to the planned doses. All preparations were stirred for about 1 h prior to use. Z-Cote Max particles were suspended in DMSO and FBS.

97. In the plate incorporation method, the particle suspensions were added directly to the mixture of agar and bacteria, and poured onto agar plates. In the pre-incubation method, the particle suspension is pre-incubated with the bacterial suspension at 37 °C for the 20 minutes using a shaker. Subsequently, 2 mL of soft agar is added and, after mixing, the samples are poured onto the agar plates. In both methods, the agar plates were incubated at 37°C for 48 h to 72 h in the dark, and the bacterial colonies were counted. Three test plates/ dose or per control were investigated.

Deviation from TG

98. For NM-113 the test was performed without metabolic activation.

Results

99. Precipitation of the NM-113 (from about 500 µg/plate), Z-COTE Max (from about 2500 µg/plate) occurred. A bacteriotoxic effect was occasionally observed in NM-113 treated cultures depending on the strain and test conditions from about 2500 µg/plate onward. A relevant increase in the number of his+ revertants was not observed in the standard plate or in the pre-incubation test in the absence (NM-113, Z-COTE Max) and presence (Z-COTE Max) of a metabolizing system. Thus, all the ZnO MNMs tested were not mutagenic under the test conditions chosen.

Studies on SWCNTs - Two studies were reported

Assay procedure

100. The studies tested two different types of MNMs: Nikkiso SWCNTs and Supergrowth SWCNTs. CNTs were suspended in water for injection, diluted with 0.3% CMC-Na solution (Nikkiso SWCNTs) or 0.1% CMC-Na (Supergrowth SWCNTs), and sonicated. The CNTs were tested in *S. typhimurium* TA 1535, TA 1537, TA 98, TA 100, and *E. coli* WP2. In addition, the Supergrowth SWCNT were tested in *S. typhimurium* strain TA97 and the Nikkiso SWCNTs in *S. typhimurium* strain TA 1537.

101. The mutation test was performed with and without metabolic activation. The following positive controls were used: 1) without S9 mixture (2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (TA100, TA98, and WP2 *uvrA*), sodium azide (TA1535) and 9-aminoacridine (TA1537)) and; 2) with S9 mixture (2-aminoanthracene (five strains)). The study was conducted using duplicate plates for each dose concentration. Different concentrations were used for Nikkiso SWCNTs (1.56 µg/plate, 3.125 µg/plate, 6.25 µg/plate, 12.5 µg/plate, 25 µg/plate, 50 µg/plate, and 100 µg/plate) and Supergrowth SWCNTs (12.5 µg/plate, 25 µg/plate, 50 µg/plate, 100 µg/plate, and 500 µg/plate).

102. The Ames test was performed with a pre-incubation method in the presence and absence of metabolic activation, via S9 mixture. In the direct method (no metabolic activation), 0.5 mL of 100 mmol/L sodium-phosphate buffer (pH 7.4) and 0.1 mL of each bacterial suspension were added to sterile tubes with distilled water, 0.1% CMC-Na, SWCNTs, or a positive control. In the metabolic activation method, S9 mixture and each bacterial suspension were added to sterile tubes with distilled water, 0.1% or 0.3% CMC-Na, SWCNTs, or a positive control. The mixtures were incubated at 37 °C with shaking (at a rate of 85 times per minute) for 20 minutes and then top agar (at 45°C) was added. The mixtures were poured into plates and incubated at 37 °C for 48 h. The number of revertant colonies was counted using a colony counter.

Results

103. No mutation induction was observed neither with nor without metabolic activation in all the tested concentrations and in all the strains.

Studies on MWCNTs - Three studies were reported.

Assay procedure

104. The studies tested three different types of MWCNTs: Baytubes, Graphistrength C100, and Mitsui-1 MWCNTs. In addition, one study was reported in which CNI MWCNTs were tested using the Ames test, but not according TG 471. The MWCNTs were suspended in various dispersants (deionised water at 10 mg/ml (Baytubes, treated with ultrasound for 30 min at 25°C), ethanol at 10 mg/mL (Graphistrength C100), or in 0.3% sodium carboxymethyl cellulose (Mitsui MWNT-7)).

105. All MWCNTs were tested in *S. typhimurium* (TA 98, TA 100, TA 1535, and TA 1537). In addition, Baytubes and Graphistrength C100 were tested in *S. typhimurium* TA 102, while Mitsui MWNT-7 in *E. coli* WP2uvrA. The applied concentrations varied between nanotubes (up to 5000 µg/plate for Baytubes; 15.6 µg/plate to 500 µg/plate for Graphistrength C100; 3.13 µg/plate to 100 µg/plate for Mitsui MWNT-7).

106. For Graphistrength C100 each strain was exposed to at least five dose-levels of the test item (three plates/dose-level). After 48 h to 72 h of incubation at 37°C, the revertant colonies were scored. The evaluation of the toxicity was performed on the basis of the observation of the decrease in the number of revertant colonies and/or a thinning of the bacterial lawn. For Mitsui MWNT-7 the pre-incubation method with and without

metabolic activation (S9 mixture) was used. Sodium azide was used as positive control. For Baytubes no further detail on exposure are provided.

Results

107. None of the tested MWCNTs showed any mutagenic activity in the bacterial reverse mutation test.

Studies on fullerenes - One study was reported

Assay procedure

108. Fullerenes (C60) were tested in *S. typhimurium* TA 1535, TA 1537, TA 98, and TA 100 without and with metabolic activation (rat liver S9 fraction). The MNMs were stirred and pulverized in Ultra Apex Mill (with carboxymethyl-cellulose sodium solution for 30 minutes). After centrifugation, the supernatant was concentrated using a 45 µm ultrafiltration membrane. The methods of application were the modified plate incorporation method under irradiation and the pre-incubation method under dark conditions. Fullerenes were applied at concentrations 50 µg/plate, 100 µg/plate, 200 µg/plate, 400 µg/plate, and 1000 µg/plate. For the test under dark conditions, each mixture was cultured for 20 minutes at 37°C with shaking. Following pre-incubation, molten top agar at 45 °C was added to the mixture and incubated for 48 h at 37 °C. For the test under irradiation, each mixture was irradiated with visible light using two fluorescent lamps (FHF32EDX-P-NU, EDXRa92; 32W, color temperature of 8000K, 400 nm to 720 nm) at 5000 lx for 1 h. Following irradiation, top agar was added and incubated for 48 h at 37 °C.

Results

109. *In vitro* bacterial mutagenicity of fullerene C60 NPs was negative regardless of metabolic activation and irradiation.

Overall summary and discussion

110. The bacterial reverse mutation test was used to assess the mutagenic potential of eight types of MNMs. In most cases, the assay was used both with and without metabolic activation (S9 mixture). None of the materials tested showed any mutagenic activity in the bacterial reverse mutation test. However, one study underlined the importance of the lack of uptake of the MNMs into bacteria, and thus the possibility of false negative results. This is in line with the conclusion of the OECD Expert Meeting on Genotoxicity of Manufactured Nanomaterials [ENV/JM/MONO(2014)34] in which one of the conclusions was that the use of Ames test (TG471) is not a recommended test method for the investigation of the genotoxicity of MNMs (OECD, 2014_[22]). The TG programme should consider modifying the applicability domain within this guideline accordingly.

3.3.2. TG 473: In Vitro Mammalian Chromosomal Aberration Test

(OECD, 2016_[23])

General introduction

111. The purpose of the *in vitro* chromosomal aberration test is to identify substances that cause structural chromosomal aberrations in cultured mammalian cells.

Assay principle

112. Mammalian cells are exposed to the test substance. After exposure the cells are treated with a metaphase-arresting substance and analysed for the presence of chromosomal aberrations. Structural aberrations may be of two types, chromosome or chromatid. Polyploidy (including endoreduplication) could arise in chromosome aberration assays *in vitro*. While aneugens can induce polyploidy, polyploidy alone does not indicate aneugenic potential and can simply indicate cell cycle perturbation or cytotoxicity.

Applicability

113. This test is used to detect chromosomal aberrations that may result from clastogenic events. The test is not designed to measure aneuploidy. An *in vitro* micronucleus test would be recommended for the detection of aneuploidy. The TG 473 mentions that for MNMs, specific adaptations of this TG may be needed but are not described in this TG.

Considerations for the choice of cell culture

114. The *in vitro* chromosomal aberration test may employ cultures of established cell lines or primary cell cultures of human or rodent origin. A variety of cell lines (e.g. Chinese Hamster Ovary (CHO), Chinese Hamster lung V79, Chinese Hamster Lung (CHL/IU), or primary cell cultures, including human (TK6) or other mammalian peripheral blood lymphocytes, can be used. The choice of the cell lines used should be scientifically justified. The cells used should be selected on the basis of growth ability in culture, stability of the karyotype (including chromosome number) and spontaneous frequency of chromosomal aberrations. At the present time, the available data do not allow firm recommendations to be made but suggest it is important, when evaluating chemical hazards, to consider the p53 status, genetic (karyotype) stability, DNA repair capacity and origin (rodent versus human) of the cells chosen for testing. The users of this TG 473 are thus encouraged to consider the influence of these and other cell characteristics on the performance of a cell line in detecting the induction of chromosomal aberrations, as knowledge evolves in this area.

115. **Note:** Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation to ensure that potential genotoxicity of liver metabolites of the tested compound is also detected (unless the cells are metabolically competent with respect to the test substances). The exogenous metabolic activation system does not entirely mimic *in vivo* conditions. Care should be taken to avoid conditions that could lead to artefactual positive results, i.e. chromosome damage not caused by direct interaction between the test chemicals and chromosomes; such conditions include changes in pH or osmolality, interaction with the medium components or excessive levels of cytotoxicity. Also for such systems the negative results do not always imply the lack of hazard potential.

General procedure

116. Cell cultures of human or other mammalian origin are exposed to the test chemical both with and without an exogenous source of metabolic activation unless cells with an adequate metabolizing capability are used. The analysis of chromosomal aberration induction should be done using cells in metaphase. It is thus essential that cells should reach mitosis both in treated and in untreated cultures.

117. At appropriate predetermined intervals after the start of exposure of cell cultures to the test chemical, the cells are treated with a metaphase-arresting substance (e.g. Colcemid® or colchicine), harvested, stained and metaphase cells are analysed microscopically for the presence of chromatid-type and chromosome-type aberrations.

Studies from the Testing Programme where TG 473 was used to test MNMs

118. The TG 473 was used in the OECD Testing Programme to test Au NPs, Ag NPs, ZnO NPs, SiO₂ NPs, fullerenes, SWCNTs, and MWCNTs.

Studies on Au MNMs - One study was reported

Assay procedure

119. The NPs were dispersed in cell culture medium and tested in CHO cells at different concentrations 0.375 nm, 0.75 nm, 1.5 nm, and 3 nm (units as described in the dossier, assumed to be nanomolar) with three replicates of each concentration. Ethylmethanesulfonate (5 mmol/L) was used as the positive control. No further details on the exposure are provided.

Deviation from the current TGs

120. The assay was performed without metabolic activation.

Results

121. No cytotoxicity was observed. Treatment with cAu NPs induced chromosomal aberrations at a frequency of between 23% □ 37% as compared to 10% in the negative control and 76% in the positive control. Under the test conditions, Au NPs induced chromosomal aberrations in CHO cells, without metabolic activation, but there was no concentration related increase in the observed aberrations.

Studies on Ag MNMs - One study was reported

Assay procedure

122. The NPs (citrate-capped Ag) were dispersed sterilised distilled water and tested in CHO-fibroblasts (CHO-K1). 20.48% of cAg NP solution was employed for this test.

123. To determine chromosome aberration test dose level, relative cell count was calculated for all cultures treated with the test substances and control substance at the 8 dose levels - 0.039 µL/mL, 0.078 µL/mL, 0.156 µL/mL, 0.313 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, and 5 µL/mL.

124. In the main test without S9 mixture, several concentrations were used for 24 h (0 µL/mL, 0.001 µL/mL, 0.003 µL/mL, and 0.005 µL/mL) and 6 h (0 µL/mL, 0.005 µL/mL, 0.010 µL/mL and 0.019 µL/mL) treatment and 18 h recovery period. The concentrations tested in the presence of S9 mixture for a 6 h treatment were: 0 µL/mL, 0.039 µL/mL, 0.078 µL/mL, and 0.156 µL/mL.

125. The positive controls used were 0.04 µg/µL of mitomycin C (in the absence of S9 mixture) and 10 µg/µL of cyclophosphamide (in the presence of S9 mixture). No further details on the exposure are provided.

126. Statistical analysis was performed to determine the increase in frequency of aberrant metaphase and polyploidy. A concentration-dependent increase or a reproducible

increase in the number of cells with chromosome aberrations was considered to be positive.

Deviation from the current TGs

127. Details on media, cell, and culture conditions, incubation time and temperature, chromosome preparation, number of metaphases analysed, individual and summary tables, and historical negative and positive control data are not reported.

Results

128. In the range finding test, following concentrations were used: with metabolic activation: 0.156 µL/mL; without metabolic activation: 0.078 µL/mL (6 h treatment), and 0.078 µL/mL (24 h treatment). Ag NPs did not induce an increase in chromosomal aberration. Under the test conditions, cAg NPs are not considered as clastogenic in CHO-fibroblast (CHO-K1) cells, with or without metabolic activation.

Studies on SiO₂ MNMs - Four studies were reported

Assay procedure

129. The studies used four different types of SiO₂ MNMs (NM-200, NM-202, NM-203, and NM-204). The SiO₂ MNMs were tested in various cell lines: V79 (NM-200), CHO (NM-202 and NM-203), and Human embryonic lung cells (WI-38) (NM-204).

130. Various protocols for the dispersion of SiO₂ MNMs were used. **NM-200** was sterilized by heating to 180°C for 1 h. The test substance was then accurately weighed, and the stock suspensions were prepared in the cell culture media with 10% or 2% FBS, respectively. To minimize agglomeration of the NM-200 particles, the stock solutions were stirred for 24 h at room temperature prior to cell incubation. This procedure could not avoid agglomeration of existing test substance aggregates, but enabled homogeneous dosing of the suspensions. Dilutions of the stock suspensions were subsequently made by using the respective incubation media, resulting in test substance suspensions at desired concentrations. Prior to cell exposure, the diluted test substance suspensions and the reference item-containing incubation media were vortexed shortly to ensure homogeneity. **NM-202, NM-203** were dispersed in DMSO. No further details were provided.

NM-200

131. The test was performed with and without metabolic activation. A short-term treatment assay (6 h) was run with and without S9 mixture, and a continuous treatment assay (24 h) was run without S9. The concentrations applied for the short-term treatment were with S9 mixture: 600 µg/mL, 1000 µg/mL, and 1500 µg/mL, while the concentrations without S9 mixture were 100 µg/mL, 200 µg/mL, 600 µg/mL, and 1800 µg/mL. For 24 h exposures, without S9 mixture, the concentrations tested were 2 µg/mL, 5 µg/mL, 16 µg/mL, and 48 µg/mL.

NM-202, NM-203

132. The test was performed with and without metabolic activation. The exposure duration was 18 h without S9 mixture and 2 h with S9 mixture. The concentrations applied for the experiments without S9 were 38 µg/mL, 75 µg/mL, 150 µg/mL, and 300 µg/mL, and for the experiments with S9 were 250 µg/mL, 500 µg/mL, 750 µg/mL, and 1000 µg/mL.

NM-204

133. The assay was performed without metabolic activation. The cells were exposed at 0.1 µg/mL, 1.0 µg/mL, and 10 µg/mL of MNMs for 24 h and 48 h.

134. At the end of the exposure period colcemid was added to the cultures 2 h prior to harvesting to collect metaphase cells. The chromosome preparations were stained with 2% Giemsa solution. Cytotoxicity was assessed by the mitotic index, i.e. percentage of cells in mitosis per 500 cells counted.

Deviation from the current TGs

135. For NM-202 and NM-203, 100 instead of 200 metaphases were scored, while for NM-204, the test was done without metabolic activation.

Results

NM-200

136. Clear concentration-dependent cytotoxicity in the short term experiments was observed for NM-200, as determined by the mitotic index. Cytotoxicity was also observed after 24 h of incubation and was more pronounced than after 4 h of incubation (≥ 500 mg/ml (4 h without S9 mixture), > 1000 µg/mL (4 h with S9 mixture), and ≥ 10 µg/ml (24 h without S9 mixture)).

NM-202 and NM-203

137. Cell proliferation began to be inhibited at 30 µg/L (-S9) and 300 µg/L (+S9). Simultaneously, the cell cycle became retarded with an accumulation of cells in the M1 phase. Neither in the control nor in the treated cultures, were the cells observed in the M3 phase (except 1 instance in the DMSO control).

138. All four tested SiO₂ MNMs (NM-200, NM-202, NM-203, and NM-204) did not induce structural chromosomal aberrations in cultured mammalian somatic cells under the test conditions used.

Studies on ZnO MNMs - One study was reported

Assay procedure

139. Z-COTE HP1 (NM-111) was tested using the *in vitro* mammalian chromosome aberration test according to OECD TG 473 under GLP conditions in comparison to the reference items Z-COTE (NM-110) and microscale ZnO (NM-113). Proliferating V79 cells were treated with different concentrations of Z-COTE HP1 (1 µg/mL, 3 µg/mL, 5 µg/mL, 10 µg/mL, 12.5 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL, and 50 µg/mL), Z-COTE (3 µg/mL, 10 µg/mL, and 12.5 µg/mL), and microscale ZnO (3 µg/mL, 10 µg/mL, and 12.5 µg/mL) of the test item and two reference items as well as negative, vehicle, and positive controls for 4 h with and without S9 mixture and for 24 h without S9 mixture.

Results

140. After a 4 h exposure period Z-COTE HP1 caused dose-dependent cytotoxicity while the highest dose decreased the mitotic index to 44.4% (with S9 mixture) and to 47% (without S9 mixture) of the negative control. There was also a decrease in mitotic index for Z-COTE (44.3%) and microscale ZnO (54.6%), primarily without S9 mixture, which was even more pronounced than for Z-COTE HP1 at identical mass concentrations. Neither the test item nor the particular reference items induced increased structural chromosome aberration under the present test conditions. Due to the low aberration frequencies in Z-COTE HP1, Z-COTE, and microscale ZnO treated cells,

which were in range of the historical negative controls, the three tested ZnO particle items were considered not to induce structural chromosomal aberrations in cultured mammalian somatic cells under the test conditions used.

Studies on fullerenes - One study was reported

Assay procedure

141. Fullerenes were dispersed in 0.1% CMC-Na. The particles were tested in Chinese Hamster lung (CHL/IU) cells, with and without metabolic activation. A short-term treatment assay (6 h) was run with and without S9 mixture and a continuous treatment assay (24 h) was run without S9 mixture. In the test under irradiation, each mixture was irradiated with visible light using two fluorescent lamps (FHF32EDXP-NU EDX Ra92; 32W, color temperature = 8000 K, 400–720 nm; Mitsubishi Electric Osram Ltd., Japan) at 5000 lx for 1 h (irradiation). Following irradiation, top agar was added and incubated for 48 hrs at 37°C. At the end of the exposure period, colcemid was added to the cultures at the final concentration of 0.2 µg/mL, 2 h prior to collecting metaphase cells. The chromosome preparations were stained with 2% Giemsa solution.

142. In a preliminary test, precipitation of C60 occurred at a concentration of 85 µg/mL without irradiation or 200 µg/mL with irradiation after treatment. Therefore, 100 µg/mL and 200 µg/mL were selected as the highest dose in the test without irradiation and the test with irradiation, respectively. Test concentrations used in the chromosomal aberration test are as follows:

- i. Short-term treatment assay: under dark conditions, the concentrations used were 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL without S9 mixture and 25 µg/mL, 50 µg/mL, and 100 µg/mL with S9 mixture, respectively. Under irradiation, the concentration used were 50 µg/mL, 100 µg/mL, and 200 µg/mL without S9 mixture and 25 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL with S9 mixture, respectively.
- ii. Continuous treatment assay: under dark conditions, the test concentrations used were 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL without S9; under irradiation, 25 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL without S9.

143. 0.1% CMC-Na was used as a negative control, and Mitomycin C, benzo[a]pyrene or acridine orange were used as positive controls.

Results

144. Fine precipitations were observed at 100 µg/mL under the dark conditions and at 200 µg/mL with S9 mixture under irradiation. No cytotoxicity was observed in the cultures treated with C60 except at 200 µg/mL with S9 mixture under irradiation. The incidence of cells with structural chromosomal aberrations was less than 5% and that of polyploid cells was 0% to 1.0%, showing no statistically significant increase at any dose compared with the vehicle control. No increase in the incidence of either type of aberration was observed at any dose regardless of metabolic activation and irradiation.

Studies on SWCNTs -Two studies were reported

Assay procedure

145. The studies used two different types of SWCNTs (Nikkiso and Super Growth). Nikkiso CNTs were suspended in water and diluted with 0.3% CMC-Na solution after sonication at each concentration. Super Growth CNTs were suspended in the distilled

water with 0.1% CMC-Na and the solution was used for the negative and vehicle control. Both SWCNTs were tested in CHL/IU cells.

146. A short term incubation of 6 h was applied in the presence and absence of the metabolic activation system (S9 mixture). In addition, a long term incubation (24 h) was done without metabolic activation. The positive controls used were mitomycin C (in the presence of S9 mixture) and benzo(a)pyrene (in the absence of S9 mixture). For Nikkiso SWCNTs the following concentrations were used: 6.25 µg/plate, 12.5 µg/plate, 25 µg/plate, 50 µg/plate, and 100 µg/plate. For Super Growth SWCNTs the following concentrations were used: 390 µg/plate, 500 µg/plate, and 1000 µg/plate.

Results

147. No cytotoxicity was observed at any concentration tested. The expressions of structural chromosomal aberration and polyploidy were not increased. In both short term tests (6 h) under the metabolism activation existence and non-existence and long term tests (24 h) under the metabolism activation non-existence, the expressions of structural chromosomal aberration and polyploidy were below 5%. The chromosome aberration was judged to be negative.

Studies on MWCNTs - Two studies were reported

Assay procedure

148. The studies used two different types of MWCNTs (Baytubes and Graphistrength C100). The MWCNTs were tested in V79 cells (Baytubes) and human lymphocytes (Graphistrength C100). Baytubes were formulated in deionised water at 10 mg/ml and treated with ultrasound for 30 min at 25 °C, while Graphistrength C100 were dispersed in ethanol. V79 cells were exposed to Baytubes (2.5 µg/mL, 5 µg/mL, and 10 µg/mL) in the absence or presence of S9 mixture for 4 h. Harvest was 18 h after the beginning of the treatment. In addition, cells treated with 10 µg/mL were harvested 30 h after the beginning of the treatment. An additional experiment was performed using continuous treatment at 2.5 µg/mL, 5 µg/mL, and 10 µg/mL for 18 h (no S9 mixture) with subsequent harvest.

149. Graphistrength C100 were tested both with and without a liver metabolizing system (S9 mixture). The treatment-concentrations were 0.78 µg/mL, 1.56 µg/mL, 3.13 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, and 50 µg/mL both with and without S9 mixture. No further information on exposure is provided.

Results

150. No cytotoxic or clastogenic effects were observed for both MWCNTs under the test conditions used.

Overall summary and discussion

151. The *in vitro* mammalian chromosomal aberration test was used to assess the mutagenic potential of seven types of MNMs. In most cases the assays were used both with and without metabolic activation (S9 mixture). The majority of MNMs were tested in the CHO cell line, but V79 cells, CHL/IU, human lymphocytes, and WI-38 cells were also used. The time of exposure was mostly 4 h (with metabolic activation) and 4 h and 24 h (without metabolic activation), but other times were also used. None of the materials tested showed any mutagenic activity in the *in vitro* mammalian chromosomal aberration

test. No information regarding potential MNMs interferences with the assays was reported.

3.3.3. TG 476: In Vitro Mammalian Cell Gene Mutation Tests using the *Hprt* and *xprt* genes

(OECD, 2016_[24])

General introduction

152. The purpose of the *in vitro* mammalian cell gene mutation test is to detect gene mutations induced by chemical substances. The xanthine-guanine phosphoribosyl transferase (XPRT) is currently less widely used than the hypoxanthine-guanine phosphoribosyl transferase (HPRT or gpt) test for regulatory purposes.

Applicability

153. It is known that some test substances may lead to false positive results in this assay. False positive results are caused by changes in the conditions such as in pH or osmolality, interaction with the medium components, or excessive levels of cytotoxicity instead of direct interactions between the test chemicals and the genetic material of the cell. Cytotoxicity should be evaluated using relative survival, i.e., cloning efficiency of cells plated immediately after treatment, adjusted by any loss of cells during treatment, based on cell count, as compared with adjusted cloning efficiency in negative controls (assigned a survival of 100%). If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve between 20 and 10% relative survival. Care should be taken when interpreting positive results only found at 10% relative survival or below. Cytotoxicity exceeding the recommended top cytotoxicity levels is considered excessive for the HPRT test. Before use of the TG on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for testing of the mixture. The TG does not contain any MNMs specific considerations.

Assay principle

154. The cell lines used in these tests measure forward mutations in reporter genes, specifically the endogenous HPRT (*Hprt* in rodent cells, *HPRT* in human cells; collectively referred to as the *Hprt* gene and HPRT test in this Guideline), and the XPRT. Mutant cells deficient in Hprt enzyme activity in the HPRT test or xprt enzyme activity in the XPRT test are resistant to the cytostatic effects of the purine analogue 6-thioguanine (TG). The Hprt (in the HPRT test) or gpt (in XPRT test) proficient cells are sensitive to TG, which causes the inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TG, whereas normal cells, which contain the Hprt (in the HPRT test) or gpt (in XPRT test) enzyme, are not.

155. The HPRT and XPRT mutation tests detect different spectra of genetic events. In addition to the mutational events detected by the HPRT test (e.g. base pair substitutions, frameshifts, small deletions and insertions) the autosomal location of the gpt transgene may allow the detection of mutations resulting from large deletions and possibly mitotic recombination not detected by the HPRT test because the *Hprt* gene is located on the X-chromosome.

Note

156. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation (usually the S9 mixture) to ensure that potential genotoxicity of liver metabolites of the tested compound is also detected (unless the cells are metabolically competent with respect to the test substances). The exogenous metabolic activation system does not entirely mimic *in vivo* conditions.

Assay procedure

157. Cells in suspension or monolayer cultures are exposed to the test chemical, both with and without an exogenous source of metabolic activation, for a suitable period of time (3 h to 6 h), and then sub-cultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. Cytotoxicity is determined by relative survival (RS), i.e., cloning efficiency measured immediately after treatment and adjusted for any cell loss during treatment as compared to the negative control. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each cell type, to allow near-optimal phenotypic expression of induced mutations (typically a minimum of 7 days to 9 days). Following phenotypic expression, mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant colonies, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. Mutant frequency is calculated based on the number of mutant colonies corrected by the cloning efficiency at the time of mutant selection.

Studies from the Testing Programme where TG 476 was used to test MNMs

158. The TG 476 was used in the OECD WPMN Testing Programme to test TiO₂ NPs, ZnO NPs, SiO₂ NPs, and MWCNTs.

Studies on TiO₂ MNMs - Four studies were reported

Assay procedure

159. The studies tested four different types of TiO₂ MNMs (NM-101, NM-103, NM-104, and NM-105). All TiO₂ NPs were tested in mouse lymphoma cells (L5178Y TK +/-). The particles were dispersed in BSA 0.05% prepared in milliQ water (2.56 mg/mL stock solution in BSA 0.05 %, sonication for 16 minutes at 10% amplitude). A single dose exposure was performed at a concentration range of 32 µg/mL, 64 µg/mL, 128 µg/mL, 256 µg/mL, 312.5 µg/mL, 625 µg/mL, 1250 µg/mL, 2500 µg/mL, and 5000 µg/mL. The duration of exposure was 24 h.

Deviation from the current TGs

160. The assay was performed without metabolic activation.

Results

161. All TiO₂ NPs resulted to be not mutagenic in L5178Y TK +/- cells at the tested doses with the *in vitro* mammalian cell gene mutation test.

Studies on SiO₂ MNMs - Seven studies were reported

Assay procedure

162. The studies used four different types of SiO₂ MNMs (NM-200, NM-201, NM-202, and NM-203). The NM-200, NM-201, NM-202, and NM-203 were tested in L5178Y TK +/- cells without metabolic activation, at concentrations 32 µg/mL, 64 µg/mL, 128 µg/mL, 256 µg/mL, 625 µg/mL, 1250 µg/mL, 2500 µg/mL, and 5000 µg/mL, exposure duration as 24 h. In addition, NM-200 was tested in L5178Y TK +/- cells in the presence of S9 mixture. In the latter study concentration 10 µg/mL, 100 µg/mL, 300 µg/mL, 900 µg/mL, 2700 µg/mL, and 5000 µg/mL were used and the times of exposure were 4 and 24 h. The CHO cell line was used to test NM-202 and NM-203 with and without metabolic activation. The concentrations applied without the S9 mixture were - S9: 10 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL, and 250 µg/mL, and with S9 mixture were 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, and 500 µg/mL. The CHO cells were incubated with the particles for 5 h.

Deviation from the current TGs

163. In some studies, the assay was performed without metabolic activation.

Results

164. The results obtained in L5178Y TK +/- cells and CHO cells were negative (no mutagenicity) for all tested SiO₂ MNMs at the tested concentrations.

Studies on ZnO MNMs - One study was reported

Assay procedure

165. Z-COTE HP (NM-111) NPs were tested using the *in vitro* mammalian cell gene mutation test according to OECD 476 in comparison to the reference items NM-110 and NM-113 under GLP conditions. The particles were dispersed in phosphate buffer prepared at a volumetric ratio of 1 to 9 with 100 µg/mL soy lecithin (1 part) and RPMI-1640 medium with 5% horse serum (9 parts). The particle suspension were added to L5178Y TK +/- cells in the presence or absence of metabolic activation at concentrations 1, 2 µg/mL, 4 µg/mL, 5 µg/mL, and 6 µg/mL (without S9 mixture) and 2.5 µg/mL, 5 µg/mL, 7.5 µg/mL, and 10 µg/mL (with S9 mixture). The cells in suspension culture were treated for 4 h. Controls included negative controls (medium, medium with soy lecithin, methyl methane sulfonate, and cyclophosphamide), vehicle control, and positive controls (with and without S9 -mixture). The concentrations were chosen based on a pre-test performed for dose range finding related to cytotoxicity.

166. The gene mutation potential of the test item Z-COTE HP1 (coated nanoscaled ZnO) was determined in comparison to the reference items Z-COTE (non-coated nanoscaled ZnO) and micro scaled ZnO. Therefore the treated cells were cultured for 6 -7 days in cell culture medium followed by a 14 days to 16 days culture period in selection medium containing 5-fluorothymidine (TFT). Finally, the mutant frequency was calculated based on the number of TFT resistant colonies evaluated microscopically. In addition the treated cells were tested for cytotoxicity (relative total growth). All experiments of this study were performed in duplicates.

Results

167. NM-111 induced increases of mutant frequency in both replicates at 6 µg/mL without S9 mixture (relative total growth: 21% compared to vehicle control) and 7.5 µg/mL with S9 mixture (relative total growth: 50% compared to vehicle control), and in one replicate at 5 µg/mL without S9-mix (relative total growth: 62% compared to vehicle

control). However, significantly increased mutant frequency was always linked to cytotoxicity. In tests using the S9 mixture, relevant increases in mutant frequency were obvious for both MNMs at 7.5 µg/mL (relative total growth: ≤ 52% compared to vehicle control) but these increases were again linked to cytotoxicity. Furthermore, slightly increased turbidity was noted for NM-111 at 10 µg/mL, for NM-110 at 7.5 µg/mL as well as for NM-113 which may influence the conduct of the test. The test results are considered as ambiguous by the author for NM-111, NM-110 as well as for NM-113 as increases in mutant frequency were always linked to cytotoxicity.

Studies on MWCNTs - One study was reported

Assay procedure

168. The MWCNTs (Graphistrength C100) were dispersed in ethanol and added at concentrations 0.625 µg/mL, 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 20 µg/mL to L5178Y TK +/- cells. Approximately (0.5 x 10⁶) cells/mL (3 h treatment) or (0.15 x 10⁶) cells/mL (24 h treatment) in 20 mL culture medium with 5% horse serum were exposed to the test or control items, in the presence or absence of S9 mixture (final concentration of S9 fraction 2%), at 37°C. Cytotoxicity was measured by assessment of adjusted relative total growth and relative suspension growth (as well as cloning efficiency following the expression time. The number of mutant clones (differentiating small and large colonies) was checked after the expression of the mutant phenotype.

Results

169. No mutagenic activity was induced by MWCNTs in L5178Y TK +/- cells.

Overall summary and discussion

170. *In vitro* mammalian cell gene mutation tests were used to assess the mutagenic potential of four types of MNMs. In most cases the assays were performed both with and without metabolic activation (S9 mixture). The L5178Y TK +/- cells were used to test all MNMs. In addition, CHO cells were used in two studies to test SiO₂ NPs. None of the materials tested showed clearly evident mutagenic activity in the *in vitro* mammalian cell gene mutation tests. No information regarding potential MNMs interferences with the assays was reported for MWCNTs, SiO₂ NPs, and TiO₂ NPs. For ZnO NPs the increased turbidity was reported at higher concentrations which could potentially influence the conduct of the test.

171. The *in vitro* mammalian cell gene mutation tests (TG 476) is considered as an alternative for the bacterial reverse mutation test (TG 471), as no report has yet identified specific limitations when testing MNMs with TG 476 [ENV/JM/MONO(2014)34].

3.3.4. TG 487: In Vitro Mammalian Micronucleus Test

(OECD, 2016_[25])

General introduction

172. The *in vitro* micronucleus test is a genotoxicity test for the detection of micronuclei in the cytoplasm of interphase cells after treatment with the test substance. The test is a method that provides a comprehensive basis for investigating chromosome damaging potential *in vitro* because both aneugens and clastogens can be detected in cells that have undergone cell division during or after exposure to the test chemical.

Micronuclei represent damage that has been transmitted to daughter cells, whereas chromosome aberrations scored in metaphase cells may not be transmitted. This TG 487 also allows for the use of protocols without cytokinesis block, provided there is evidence that the cell population analysed has undergone mitosis. As micronuclei may arise from lagging chromosomes, there is the potential to detect aneuploidy-inducing agents that are difficult to study in conventional chromosomal aberration tests, e.g. OECD TG 473. However, the *in vitro* micronucleus test as described in TG 487 does not allow for the differentiation of substances inducing changes in chromosome number and/or ploidy from those inducing clastogenicity without special techniques such as Fluorescence *In Situ* Hybridization (FISH).

173. To analyse the induction of micronuclei, it is essential that mitosis has occurred in both treated and untreated cultures. The most informative stage for scoring micronuclei is in cells that have completed one mitotic phase during or after treatment with the test chemical.

Applicability

174. Some test substances tested in the *in vitro* micronucleus assay can give false positive results which do not reflect the genotoxicity of the test chemicals. Such conditions include changes in pH or osmolarity, interaction with the cell culture medium or excessive levels of cytotoxicity. The TG mentions that for MNM specific adaptations of this TG are needed but they are not described.

Assay principle

175. The mammalian cell micronucleus *in vitro* test is robust and can be conducted in a variety of cell types. The test may employ cultures of cell lines or primary cell cultures, of human or rodent origin. It is recommended that cell types with a stable and defined background frequency of micronucleus formation be used. Cell cultures of human or other mammalian origin are exposed to the test chemical both with and without an exogenous source of metabolic activation unless cells with an adequate metabolising capability are used. Micronuclei may originate from acentric chromosome fragments (i.e. lacking a centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The TG 487 allows the use of protocols with and without the actin polymerisation inhibitor cytochalasin B. The addition of cytochalasin B prior to mitosis results in cells that are binucleate and therefore allows for the identification and analysis of micronuclei in only those cells that have completed one mitosis. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation unless the cells are metabolically competent with respect to the test substances. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions.

Assay procedure

176. During or after exposure to the test chemical, the cells are grown for a period sufficient to allow chromosome damage or other effects on cell cycle/cell division to lead to the formation of micronuclei in interphase cells. For induction of aneuploidy, the test chemical should ordinarily be present during mitosis. Harvested and stained interphase cells are analysed for the presence of micronuclei. Ideally, micronuclei should only be scored in those cells that have completed mitosis during exposure to the test chemical or during the post-treatment period, if one is used. In cultures that have been treated with a

cytokinesis blocker, this is easily achieved by scoring only binucleated cells. In the absence of a cytokinesis blocker, it is important to demonstrate that the cells analysed are likely to have undergone cell division, based on an increase in the cell population, during or after exposure to the test chemical. For all protocols, it is important to demonstrate that cell proliferation has occurred in both the control and treated cultures, and the extent of test chemical-induced cytotoxicity or cytostasis should be assessed in all of the cultures that are scored for micronuclei.

Studies from the Testing Programme where TG 487 was used to test MNMs

177. The TG 487 was used in the OECD WPMN Testing Programme to test SiO₂ and TiO₂ NPs.

Studies on SiO₂ MNMs - Twenty-two studies were reported

Assay procedure

178. The studies used four different types of SiO₂ NPs (NM-200, NM-201, NM-202, and NM-203). All four SiO₂ MNMs were tested in five different cell cultures: human bronchial epithelial (16-HBE) cell line, A549 cell line, BEAS-2B cell line, Caco-2 cell line, and human primary peripheral blood lymphocytes.

179. All particles were dispersed in BSA 0.05% prepared in milliQ water (2.56 mg/mL stock solution in BSA 0.05%, sonication for 16 minutes at 10% amplitude).

180. The applied concentrations varied between studies. The lowest concentration range tested was 4 µg/mL, 8 µg/mL, 16 µg/mL, 32 µg/mL, and 64 µg/mL. The highest concentration range used was 64 µg/mL, 128 µg/mL, 256 µg/mL, 312.5 µg/mL, 625 µg/mL, and 1250 µg/mL. The times of incubation of the cells with MNMs were: 16-HBE - 31 h, A549 - 48 h, BEAS-2B - 48 h, Caco-2 - 52 h, and human primary lymphocytes - 30 h.

181. In most of the studies the test was performed with addition of cytochalasin B. In 16-HBE, A549, and BEAS-2B cell lines, and human primary lymphocytes, cytochalasin B was added 6 h after addition of the MNMs, while in undifferentiated Caco-2 cell line cytochalasin B was added 24 h after addition of the MNMs. Cytochalasin B was not used in two studies (NM-200 and NM-202 tested in 16-HBE cells). All tests were performed without metabolic activation.

Results

NM-200

182. No cytotoxic effects were found in 16-HBE cells, Caco-2 cells, A549 cells and human lymphocytes at any concentration tested. Cytotoxicity was observed in BEAS-2B cells at concentrations > 256 µg/ml. The particles did not induce aneugenic/clastogenic damage in 16-HBE cells, A549 cells, BEAS-2B cells, human lymphocytes. However, in 2 out of 3 experiments, a statistical dose-dependent increase in the frequency of binucleated cells with micronuclei was observed in Caco-2 cells with the cytokinesis-block micronucleus assay. It was concluded that NM-200 is genotoxic and induces chromosomal damage at the highest doses *in vitro*.

NM-201 and NM-202

183. The particles did not induce chromosomal damage in 16-HBE, BEAS-2B, and human lymphocyte cells. However, in 2 out of 3 experiments, a statistical dose-dependent

increase in the frequency of binucleated cells with micronuclei was observed in A549 and Caco-2 cells with the cytokinesis-block micronucleus assay. It was concluded that NM-201 is genotoxic at the highest doses *in vitro*.

NM-203

184. The particles did not induce chromosomal damage in 16-HBE cells and human lymphocytes. Conflicting results were obtained in A549, Caco-2, and BEAS-2B cells, where the particles were negative in some experiments, which in other experiments a dose-dependent increase in the frequency of binucleated cells with micronuclei was observed.

Studies on TiO₂ MNMs - Twenty-six studies were reported

Assay procedure

185. The studies used four different types of TiO₂ NPs (NM-102, NM-103, NM-104, and NM-105). All four TiO₂ MNMs were tested in six different cell cultures: 16-HBE, A549, BEAS-2B cell lines, undifferentiated Caco-2 cell line, human primary peripheral blood lymphocytes, and normal human keratinocytes (NHK).

186. All particles were dispersed in BSA 0.05% prepared in milliQ water (2.56 mg/mL stock solution in BSA 0.05%, sonication for 16 minutes at 10% amplitude). The exposure doses used were from 2 µg/mL to 512 µg/mL but the applied concentrations ranges varied between studies. The times of incubation of the cells with MNMs were: 16-HBE – 41 h, A549 – 24 h, BEAS-2B – 48 h, Caco-2 – 52 h, human primary lymphocytes – 30 h.

187. In most of the studies, except the ones performed with the human bronchial 16-HBE cell line, the test was run with addition of cytochalasin B. In experiments with the A549 cells, BEAS-2B cell line, human primary lymphocytes, and NHK cells, cytochalasin B was added 6 h after addition of the MNMs, while in Caco-2 cell line cytochalasin B was added 24 h after addition of the MNMs. All tests were performed without metabolic activation.

Results

NM-102

188. The MNM did not induce aneugenic/clastogenic damage in 16-HBE, A549, and Caco-2 cells at the doses tested. The test in BEAS-2B cells was performed in 6 laboratories. No effect was observed for 4 out of 6 laboratories but one laboratory showed a positive response (increase in micronucleus frequency in binucleated cells) and other laboratory showed an equivocal response with an increase in micronuclei observed only at some doses. A dose-dependent increase in the frequency of binucleated cells with micronuclei was observed in NHK cells.

NM-103

189. The MNM did not induce aneugenic/clastogenic damage in 16-HBE, A549 cells, BEAS-2B, and Caco-2 cells at the doses tested with the micronucleus assay. Interestingly, the particles induced damage at the lower concentrations tested (5 µg/mL and 45 µg/mL, but not 15 µg/ml) in the human blood lymphocytes. A dose-dependent increase in the frequency of binucleated cells with micronuclei was observed in NHK cells.

NM-104

190. The MNM did not induce aneugenic/clastogenic damage in 16-HBE, A549, and Caco-2 cells at the doses tested. Interestingly, the particles induced damage at the lower concentrations tested (15 µg/mL and 45 µg/mL) in the human blood lymphocytes. A dose-dependent increase in the frequency of binucleated cells with micronuclei was observed in NHK cells.

NM-105

191. The MNM did not induce aneugenic/clastogenic damage in 16-HBE, BEAS-2B, A549, and Caco-2 cells, and human lymphocytes and at the doses tested. A dose-dependent increase in the frequency of binucleated cells with micronuclei was observed in NHK cells.

Overall summary and discussion

192. The *in vitro* mammalian micronucleus test was used to assess the mutagenic potential of two types of MNMs. In all cases the assay was used without metabolic activation. All MNMs were tested in 16-HBE cells, A549 cells, BEAS-2B cells, and human primary lymphocytes. In addition, all TiO₂ MNMs were tested in NHK cells. The time of exposure was between 30 h and 52 h and varied between cell lines but was the same for the same cell line. In most tests cytokinesis was blocked using Cytochalasin B. Cytochalasin B was added to the cells 6 h after addition of the MNMs in all cell lines except Caco-2 cells in which case it was added 24 h after addition of MNMs.

193. None of the tested particles induced damage in 16-HBE cells. All tested TiO₂ particles induced a dose-dependent increase in the frequency of binucleated cells with micronuclei observed in NHK cells. For the other cell lines, the results of the micronucleus tests with SiO₂ and TiO₂ particles varied. Interestingly, in two studies TiO₂ particles induced damage at the lower concentrations tested (15 µg/mL and 45 µg/mL) in the human blood lymphocytes, which was not observed at higher concentrations. No information regarding potential MNMs interferences with the assays was reported.

194. It has to be stressed that the OECD Expert meeting on Genotoxicity of Manufactured Nanomaterials [ENV/JM/MONO(2014)34] (OECD, 2014_[22]), concluded that “the extent of cellular uptake is a critical factor to consider when interpreting test results. In some circumstances, a lack of uptake in a mammalian cell may indicate a low intrinsic hazard from a direct genotoxicity perspective.” It also recommended that “the test guidelines program should consider modification of the *in vitro* micronucleus assay to recommend, where cyto B is used, its addition using a post-treatment or delayed co-treatment protocol, in order to ensure a period of exposure of the cell culture system to the nanomaterial in the absence of cyto B”. Moreover, the Scientific Committee on Consumer Safety “Guidance on the safety assessment of nanomaterials in cosmetics” (2012) (SCCS (Scientific Committee on Consumer Safety), 2012_[20]) states that “cytochalasin B, which is often used in to inhibit cytokinesis, may inhibit endocytosis and hence has been suggested to lead to false negative outcomes with particles (Landsiedel et al., 2009). For several types of nanoparticles (e.g. titanium dioxide, multi-walled carbon nanotubes), the microscopic evaluation of cytokinesis-block proliferation index and micronucleus identification was found to be inappropriate at high testing concentrations due to the overload of agglomerates (Corradi et al., 2011).”

3.3.5. Comet assay (single-cell gel electrophoresis)

General introduction

195. The Comet assay (single-cell gel electrophoresis) is a simple and sensitive method for measuring DNA damage and repair in individual eukaryotic cells. The assay detects single and double-stranded DNA breaks by measuring the migration of DNA from individual nuclei following alkaline treatment. It is frequently used for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. The alkaline Comet assay modified by additional use of lesion-specific endonucleases, such as formamido-pyrimidine-DNA glycosylase (FPG) and endonuclease III (ENDOIII, also known as Nth), can detect DNA bases with oxidative damage. The results from the Comet Assay can give an indication of potential genotoxicity of environmental chemicals.

Assay principle

196. The assay involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline conditions, and electrophoresis of the suspended lysed cells. The term "Comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet. The likely basis for this is that loops containing a break lose their supercoiling and become free to extend toward the anode.

General procedure

197. Cells treated with a test substances are embedded in agarose on a microscopic slide and lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. In the modified Comet assay, the slides are then treated with lesion-specific endonucleases, for detection of oxidative DNA-damage. This is followed by electrophoresis at high pH and visual analysis with staining of DNA. Electrophoresis results in structures resembling comets observed by fluorescence microscopy. The fluorescence intensity and length of the comet tail relative to the head reflects the number of DNA breaks and thus the extent of DNA damage. This can be determined manually by identification of comets and measurement of the fluorescence intensity using appropriate software, or automatically using a high-content imaging platform.

Studies from the Testing Programme where the Comet assay was used to test MNMs

198. The Comet assay was used in the OECD WPMN Testing Programme to test TiO₂ and SiO₂ NPs and SWCNTs.

Studies on TiO₂ MNMs: 28 studies were reported

Assay procedure

199. The TiO₂ MNMs were tested in various cell lines: NM-102, NM-103, NM-104, and NM-105 were tested in EpiDerm™, 16-HBE cells, A549 cells, Caco-2 cells, and normal human epidermal keratinocytes (NHEK). NM-101, NM-102, NM-103, NM-104, and NM-105 were tested in BEAS-2B cells and NM-105 was tested in V79. In most studies the TiO₂ NPs were dispersed in 0.05% BSA prepared in milliQ water (2.56 mg/mL stock solution in 0.05% BSA, sonication for 16 minutes at 10% amplitude).

200. The applied concentrations varied between studies and between cell lines. The lowest concentration range tested was 1 µg/mL, 10 µg/mL, 100 µg/mL. The highest concentration range used was 5 µg/mL, 100 µg/mL, and 256 µg/mL. For the EpiDerm™ model the concentrations 82 µg/cm², 164 µg/cm², and 246 µg/cm² were applied.

201. The times of incubation of the cells with MNMs were in most cases 3 h and 24 h, while for the EpiDerm™ model it was 72 h.

Results

NM-102

202. No genotoxicity was observed in the EpiDerm™ model treated with NM-102. In BEAS-2B cells the NM-102 induced genotoxic effects at both exposure times. In 16-HBE cells and A549 cells the effects were observed only after 3 h and not after 24 h of exposure. For the Caco-2 and NHEK cells the results were ambiguous.

NM-103

203. NM-103 did not induce DNA breaks in EpiDerm™, 16-HBE cells, A549 cells and BEAS-2B cells. In Caco-2 cells a positive response with the alkaline Comet assay was observed only after 24 h, while in NHEK at both 3h and 24h exposure times.

NM-104

204. NM-104 did not induce DNA strand breaks in all cell cultures except NHEK, where the result was ambiguous since the increase in the % Tail DNA at both 3h and 24h exposure was observed at one dose only.

NM-105

205. No genotoxicity was observed in the EpiDerm™ model and BEAS-2B cells treated with NM-105. NM-105 induced DNA strand breaks in 16-HBE and Caco-2 cells following 24 h (but not 3 h). In A549 cells the effects were observed only after 3 h and not after 24 h of exposure. In V79 cells NM-105 induced a genotoxic effect (significant compared to the control) at 100 mg/L after 24 h exposure.

206. No results are reported for NM-101 in the dossier.

Studies on SiO₂ MNMs - 18 studies were reported

Assay procedure

207. The SiO₂ MNMs were tested in various cell lines: NM-200, NM-201, NM-202, and NM-203 were tested in A549, BEAS-2B, and Caco-2 cells, NM-201, NM-202, and NM-203 were tested in 16-HBE cells, and NM-200 was tested in primary rat alveolar macrophage.

208. In several studies (12/18) both the alkaline Comet assay and the FpG-modified Comet assay were used. The procedure for dispersion is reported in 3 out of 18 studies. SiO₂ NPs were dispersed in 0.05% BSA prepared in milliQ water (2.56 mg/mL stock solution in 0.05% of BSA followed by sonication for 16 minutes at 10% amplitude).

209. The applied concentrations varied between studies and between cell lines. The lowest concentration range tested was 19 ng/mL, 95 ng/mL, and 475 ng/mL. The highest concentration range used was 2.56 µg/mL, 25.6 µg/mL, 256 µg/mL, and 512 µg/mL. In many studies the concentrations are reported both in µg/mL and in µg/cm². The times of

incubation of the cells with MNMs were in most cases 3 h and 24 h. For the primary rat alveolar macrophages the incubation times were 4 h and 24 h.

Results

NM-200

210. No cytotoxic effects were observed in any of the cell lines used. The observed genotoxic effects of NM-200 varied between studies. In A549 cells the nanomaterial induced equivocal genotoxic response in A549 cells following 3 h treatment and was not genotoxic following 24 h treatment at the tested dose with the alkaline Comet assay, while in a second study in the same cell line it induced a positive response in the Comet assay at 3 h at 2 doses (256 µg/mL and 512 µg/mL), and an equivocal response at 24 h with an increase at the lowest dose (2.56 µg/mL). NM-200 did not induce oxidative DNA damage in A549 cells at both 3 h and 24 h at the tested dose with the FpG-modified Comet assay.

211. In BEAS-2B cells NM-200 induced DNA strand breaks at 2 doses following 3 h treatment with the alkaline Comet assay and equivocal oxidative DNA damage following 3 h treatment at one dose only (256 µg/ml) in the FpG-modified alkaline Comet assay.

212. In Caco-2 cells, NM-200 induced DNA strand breaks at 2 doses following both 3 h (25.6 µg/mL and 256 µg/mL) and 24 h (256 µg/mL and 512 µg/mL) treatment with the alkaline Comet assay. Oxidative DNA damage was detected with the FpG-modified Comet assay after treatment with NM-200 for 24 h at 3 doses (2.56 µg/mL, 256 µg/mL, and 512 µg/mL). An equivocal response at one dose (256 µg/mL) after 3 h exposure was detected with the FpG-modified Comet assay. NM-200 did not induce DNA damage in human bronchial 16-HBE cells and in primary rat alveolar macrophages.

NM-201

213. The observed genotoxic effects of NM-201 varied between cell lines. In A549 cells the Comet assay was positive after treatment with the highest doses (256 µg/mL and 512 µg/mL) for at 3 h, while an equivocal response was observed at 24 h with an increase in the % tail DNA only at the lowest dose (2.56 µg/mL). In BEAS-2B cells NM-201 induced an equivocal response at 3 h with an increase in the % tail DNA at one dose (256 µg/mL). The cells were not treated for 24 h.

214. Contrary to the A549 cells, in Caco-2 cells the results of the Comet assay were negative in cells treated for 3 h, while an equivocal response was observed in cells treated for 24 h but only at one dose (25.6 µg/mL). NM-201 did not induce DNA damage in 16-HBE cells.

NM-202

215. The observed genotoxic effects of NM-202 varied between cell lines. In A549 cells, the Comet assay was positive after treatment with 2 doses (25.6 µg/mL and 256 µg/mL, but not 512 µg/mL) for at 3 h, while an equivocal response was observed at 24 h with an increase in the % tail DNA only at one dose. In BEAS-2B cells NM-202 induced a positive response at 3 h at 3 doses (2.56 µg/mL, 25.6 µg/mL, and 256 µg/mL). The cells were not treated for 24 h. An equivocal response at only one dose (25.6 µg/mL) was observed in Caco-2 cells treated for 3 h and 24 h. NM-202 did not induce DNA damage in 16-HBE cells.

NM-203

216. Like the other SiO₂ NPs tested, NM-203 did not induce DNA damage in 16-HBE cells.

217. In A549 cells the NM-203 did not induce genotoxicity at 3 h, while the results of the Comet assay were positive at 24 h with an increase in % tail DNA at 2 doses (25.6 µg/mL and 256 µg/mL, but not 512 µg/mL). In BEAS-2B cells and in Caco-2 cells the NM-203 induces a positive response after 3 h treatment with three doses (2.56 µg/mL, 25.6 µg/mL, and 256 µg/mL) and two doses (2.56 µg/mL and 25.6 µg/mL) respectively, while the results at 24 h were not reproducible (negative in 3 experiments, positive in 3 experiments).

Studies on SWCNTs - 7 studies were reported

Assay procedure

218. The studies tested six different types of SWCNTs: CNI, EliCarb, Sigma-Aldrich, NIST, Heji, and COCC SWCNTs. Each type of SWCNT was tested in a different cell lines: V79 (CNI SWCNT), Mouse FEI-MML epithelial cell line (EliCarb SWCNT), BEAS-2B cells (Sigma-Aldrich CNT), RAW 264.7 (Sigma-Aldrich CNT), normal human mesothelial cells (NIST SWCNT), malignant human mesothelial cells (NIST SWCNT), human peripheral blood lymphocytes (Heji SWCNT), primary mouse embryo fibroblasts (COCC SWCNT).

219. The methods of dispersion differed significantly between the types of SWCNTs tested (see Annex for details). There was a great variability in the number of doses applied, the concentrations ranges used and the dose metrics (µg/cm² or in µg/mL). Only in one case the concentrations are reported both in µg/cm² and in µg/mL. The times of incubation with MNMs varied between cell types (2 h in case of Raw 264.7 cells up to 72 h for BEAS-2B cells treated with Sigma-Aldrich CNT).

Results

220. CNI SWCNT induced DNA damage in V79 after only 3 h of incubation with 96 µg/cm² of SWCNTs. EliCARB SWCNTs did not increase the level of strand breaks in mouse FEI-MML epithelial cell line, but significantly increased the level of FpG sensitive sites/oxidized purines as determined by the Comet assay. Sigma-Aldrich CNTs induced a dose-dependent increase in DNA damage in BEAS-2B cells. After the 48 h and 72 h treatments with CNTs, the effect was also very clear; a statistically significant increase in DNA damage was observed at all tested doses, with a significant dependence on dose. In RAW 264.7 the % tail DNA was increased in a Comet assay after treatment with 10 µg/mL and 100 µg/mL of Sigma-Aldrich CNTs for 24 h.

221. In normal and malignant mesothelial cells exposed to 25 µg/cm² or 50 µg/cm² of NIST SWCNTs for 24 h, DNA damage was induced in both cell types at both doses. Exposure of NM cells to 25 or 50 µg/cm² SWCNTs for 24 hr resulted in a 5.2- and 6.6-fold increase in DNA tail length migration, respectively. In contrast, exposure of MM cells to the same mass concentrations of crocidolite for 24 h caused an increased DNA tail migration of 7.9- and 11.1-fold, respectively. Co-incubation of MM cells with SWCNTs (25 µg/cm²) and catalase (100 U/mL), SOD (100 U/mL), or deferoxamine (1 mM; an iron chelator) for 24 hr resulted in a 35%, 30%, and 32% decrease in DNA damage, respectively.

222. No genotoxic effects were found in the Comet assay in human leukocytes following 6 h treatment with Heji SWCNT at concentrations of 0.5 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL.

223. Exposure of primary mouse embryo fibroblast (BALB/c mouse) to COCC SWCNTs (5 µg/mL or 10 µg/mL) for 24 h resulted in increase of % tail DNA, tail length, and tail movement.

Overall summary and discussion

224. With the Comet assay, the responses were largely negative or equivocal in the tested cells for most of the investigated MNs. When positive results were obtained, in most cases, dose response relationship could not be established which makes it difficult to conclude on the *in vitro* genotoxicity of the MNs tested. Moreover, the large variability in all the experimental conditions (including differences in cell lines used, concentrations tested, times of exposure, and dispersion protocols) makes the comparability of the results from the various studies practically impossible. It should be noted that in contrast to other *in vitro* genotoxicity studies, the DNA damage picked up by the Comet assay may be repaired at later cell cycles. Therefore, the results from the Comet Assay can only give an indication of potential genotoxicity of environmental chemicals.

3.3.6. Double-Strand Breaks (DSB) assay (Histone H2AX phosphorylation)

General introduction

225. DNA double-strand breaks (DSBs) are formed as a result of genotoxic insults and are among the most serious types of DNA damage. One of the earliest molecular responses following DSB formation is the phosphorylation of the histone H2AX, giving rise to γ -H2AX. Many copies of γ -H2AX are generated at DSBs and can be detected *in vitro* using well-established immuno-histochemical methods.

Assay principle

226. The detection of γ -H2AX relies on immunological techniques using specific monoclonal and/or polyclonal antibodies against the H2AX C-terminal phosphorylated peptide. Total γ -H2AX levels can be measured either in cell and/or tissue lysates or directly in cells and tissues. In the first approach, the techniques establish the overall γ -H2AX levels in lysates by using immunoblotting or the enzyme-linked immunosorbent assay (ELISA). In the second approach, γ -H2AX levels are measured directly in cell nuclei by microscopy or fluorescence-activated cell sorting (FACS). Large numbers of γ -H2AX molecules formed at DNA break sites create bright foci that allow detection of individual DSBs, making foci counting the most sensitive assay to detect DNA damage. In the OECD Testing Programme the method based on microscopic detection and counting of γ -H2AX foci was used.

General procedure (as used in the OECD Testing Programme)

227. Cells are exposed to the test substance for a defined period of time. The cells are then fixed, permeabilised and stained with specific monoclonal or polyclonal primary antibodies anti- γ -H2AX, followed by the secondary fluorescent antibody. Florescent γ -H2AX foci are detected microscopically and can be quantified using automatic image analysis systems.

Studies from the Testing Programme where Double Strands Breaks (DSB) assay was used to test MNMs

228. The DSB assay was used in the OECD WPMN Testing Programme to test SWCNTs.

Studies on SWCNTs: Two studies were reported

Assay procedure

229. Arc SWCNTs, amide-functionalised SWCNTs and NIST SWCNTs were tested. Arc SWCNTs and amide-functionalised SWCNTs were suspended in dimethylsulfoxide (DMSO) and ultrasonically dispersed for 30 minutes. Exponentially growing normal human dermal fibroblasts (HDMEC) were seeded on poly-lysine glass-slides for 24 h. After that time cells were treated with four different doses of carbon nanotubes (25 $\mu\text{L}/\text{mL}$, 50 $\mu\text{L}/\text{mL}$, 100 $\mu\text{L}/\text{mL}$, and 150 $\mu\text{L}/\text{mL}$). The repair kinetics of induced DSBs was measured after 24 h. Cells were fixed with 2% paraformaldehyde, permeabilised with 0.2% Triton X-100, and stained with the primary antibody and an FITC-labelled secondary antibody. Cell nuclei were co-stained with PI. Foci were counted using an epifluorescent microscope Axiomager AI (Zeiss) and the program ImageJ.

230. For NIST SWCNTs no details on the dispersion procedure are provided. Normal and malignant mesothelial human cells were cultured in black-wall/clear-bottom microplates and exposed to 25 $\mu\text{g}/\text{cm}^2$ or 50 $\mu\text{g}/\text{cm}^2$ SWCNTs for 24 h. H2AX phosphorylation was detected according to the manufacturer's protocol of the kit (Millipore, Billerica, MA).

Results

231. In the fibroblast cell line the same dose of Arc SWCNTs induced 2.7-fold higher number of γ -H2AX foci than in a non-treated control. Amide functionalized SWCNTs also induced an increased number of γ -H2AX foci, 3.2-fold higher than the control. Exposure of NM and MM cells to 25 $\mu\text{g}/\text{cm}^2$ or 50 $\mu\text{g}/\text{cm}^2$ NIST SWCNTs resulted in a nominal increase in phosphorylation of H2AX, which was moderately higher in MM cells. The same concentrations of crocidolite (positive control) induced a significantly greater phosphorylation in both cell types.

Overall summary and discussion

232. There are several issues with interpreting the results from the reported studies to assess the genotoxicity potential of SWCNTs including, 1) each study used different cell line and SWCNTs from different sources and with different surface modifications; 2) the SWCNTs concentrations are expressed using different dose-metric ($\mu\text{g}/\text{cm}^2$ or $\mu\text{L}/\text{mL}$); 3) the first study did not use a positive control making it difficult to determine the biological relevance of the increase in the number of foci; and 4) the reports do not mention any problems of interactions of the MNMs with the assay but it could be assumed that if MNMs with autofluorescence are tested, they could interfere with the quantification of foci. All these issues make the comparison of the results impossible.

3.3.7. Literature Survey of Genotoxicity Assays

233. A literature search was performed to give an overview which *in vitro* genotoxicity assays have been used to test MNMs after the culmination of the Testing Programme. PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) was used to retrieve the abstracts of the

publication, using search keywords as listed in the notes to Table 4. The search was performed on the 6th December 2016. Table 4 reports the numbers of publication per MNM and per assay. The publication list (ANNEX) was limited to MNM types and genotoxicity assays used in the OECD Testing Programme.

Table 4. Number of publications where *in vitro* genotoxicity assays were used to test the MNMs (2010-2016)

Assay	Ag	Au	SiO ₂	TiO ₂	ZnO	CeO	SWCNTs	MWCNTs	Fullerenes
AMES test ¹	3	0	0	3	1	0	3	3	2
<i>In vitro</i> mammalian chromosomal aberration test ²	2	1	4	2	3	0	5	7	3
Mammalian cell gene mutation test (Hprt and xprt genes) ³	1	0	1	4	0	0	2	2	0
<i>In vitro</i> micronucleus test ⁴	9	2	12	19	11	1	7	12	3
<i>In vitro</i> Comet assay ⁵	7	2	4	16	7	3	4	5	1
DNA double strands break assay (H2AX phosphorylation) ⁶	1	0	1	6	3	0	1	1	0

Source: 1 PubMed search: AMES test nanomaterials genotoxicity
 2 PubMed search: chromosomal aberration *in vitro* nanomaterials
 3 PubMed search: HPRT genotoxicity nanomaterials
 4 PubMed search: micronucleus *in vitro* genotoxicity nanomaterials
 5 PubMed search: Comet assay *in vitro* genotoxicity nanomaterials
 6 PubMed search: H2AX genotoxicity nanomaterials

3.4. Cytotoxicity

234. A number of cytotoxicity assays were used under the OECD Testing Programme (see Table 5). As evident from table 5, the assays were not used consistently for all the MNMs. Although TGs do not exist for the cytotoxicity assays applied in the Testing Programme, their use and applicability in testing the overt toxicological effects of MNMs cannot be denied.

Table 5. The cytotoxicity assays used in the OECD Testing Programme

Assay	ZnO	MWCNTs	Au	Dendrimers	SWCNTs	Fullerene	SiO ₂	CeO ₂	TiO ₂
ATP CellTiter Glo			y						
Neutral red	y			y					y
LDH release		y	y	y	y	y	y	y	y
MTT				y	y	y	y	y	y
XTT			y						
Cell impedance			y						y
Trypan Blue					y				
BrDU					y				
Alamar Blue					y				
WST-1					y		y	y	
Live/dead cell counting					y				y
Colony forming efficiency		y						y	

Source: OECD Testing Programme of Manufactured Nanomaterials - Dossiers and Endpoints (OECD, 2015^[26])

3.4.1. Literature Survey of Cytotoxicity Assays

235. Since the completion of the Testing Programme, these cytotoxicity assays have been used in a number of studies to test MNMs. Table 6 shows the cytotoxicity assays and the number of studies that they have been used to test the MNMs from the current literature. PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) was used to retrieve the abstracts of the publication, using search keywords as listed in the notes to Table 6. The search was performed on 25th February 2016. Based on the information in Table 6 it is evident that there are certain assays that are consistently being used for all types of MNMs (e.g. LDH release, MTT, and cell impedance assay) while others have not been used much. It may therefore be helpful to assess the applicability of and develop standardized protocols for conducting the assays that have been used extensively to assess the overt toxicity of MNMs. Work is currently underway at the OECD and ISO TC 229 to develop standards and guidance for cytotoxicity assays.

Table 6. The number of publications where cytotoxicity assays used in the Testing Programme were used to test MNMs in the past 5 years (2010-2016)

Assay	ZnO	MWCNTs	Au	Dendrimers	SWCNTs	Fullerene	SiO ₂	CeO ₂	Ag	Nanoclay	TiO ₂
ATP CellTiter Glo	0	0	0	0	0	0	0	0	0	0	0
Neutral red uptake	2	2	1	1	2	4	1	1	4	1	2
LDH release	27	13	28	12	10	37	32	3	33	0	33
MTT	43	16	95	56	14	46	72	8	100	2	37
XTT	0	0	7	1	0	0	2	0	11	0	6
Cell impedance	11	12	41	1	10	62	16	2	13	0	33
Trypan Blue	6	4	13	0	4	10	5	1	16	0	9
BrDU	0	2	1	0	2	5	2	0	4	0	1
Alamar Blue	1	5	4	3	3	12	4	1	8	0	2
WST-1	6	4	5	2	2	10	5	0	7	0	6
Live/dead cell counting	0	0	1	0	0	0	0	0	1	0	0
Colony forming	10	4	1	0	3	11	8	1	66	0	9

Source: PubMed search results as of February 25, 2016

Filters activated: published in the last 5 years

PubMed search keywords: [MNM NAME] [ASSAY NAME] (e.g. zinc oxide nanomaterials ATP CellTiter Glo assay)

4. GENERAL OBSERVATIONS AND IDENTIFICATION OF POTENTIAL NEXT STEPS

4.1. General observations from screening of the *in vitro* assays used under the Testing Programme

- A number of *in vitro* methods were used in the OECD Testing Programme to assess MNMs, however, many *in vitro* data reported were not generated using OECD TGs.
- A number of *in vitro* endpoints have been assessed, but a full set of *in vitro* assays for a specific MNM is not available. For instance, for SWCNTs and MWCNTs, there is a lot of information on genotoxicity but very limited data on other endpoints such as e.g. skin absorption. On the other hand, a number of dermal absorption studies have been reported for ZnO. The choice of assays to be used for testing a particular MNMs was often based on potential route of exposure.
- The existing *in vitro* TGs have not been used extensively (e.g. skin sensitization and irritation). For instance, a number of TGs are available for assessing dermal effects (e.g. skin irritation (TG 439), skin sensitization (TG 442D), skin corrosion (TG 431), and skin absorption (TG 428)) but very few studies have applied them.
- There are many inconsistencies and omissions within the dossiers related to the use of existing OECD TGs, representation of dose and dose metrics, physico-chemical parameters assessed, consideration of MNM interference with assay parameters, and protocols used (e.g. sample preparation, cell types used, dose-ranges applied, time of exposure, use of positive/negative controls, use of metabolic activation systems), including inconsistencies in reporting.
- Characterization of MNMs in *in vitro* conditions (e.g. in culture media) and cellular uptake have not been reported.
- No *in vitro* data based on TGs has been reported for nanoclay and dendrimers and no *in vitro* cytotoxicity data has been reported for Ag and nanoclay within the Testing Programme.

4.2. Potential next steps

- Under the Testing Programme, one TG 428 and 2 non-TG studies were reported to assess the dermal absorption of ZnO MNMs. In addition to the Testing Programme, several studies have been reported that tested many different kinds of MNMs (including TiO₂, ZnO, SiO₂, Au, and Ag) using intact skin. Based on TG 428 and the reported studies, there is no apparent limitation in the application of the TG 428 to MNMs. However, some critical factors for the evaluation of skin absorption tests with nanomaterials were not addressed in detail in these studies, and might need to be further explored. These include the optimum exposure duration and time of sampling/evaluation, choice of the exposure concentrations (i.e. stable dispersions without material aggregation should be used), the

compatibility of the receptor fluid with MNMs need to be further explored, evaluation of the MNMs cellular uptake, the influence of mechanical process in particles translocation. Thus, the applicability of TG 428 (Skin absorption: *in vitro* method) should be further investigated. Applicability of TG 431 (*In vitro* skin corrosion: reconstructed human epidermis (RHE) test method) should be further discussed. Under the Testing Programme, four TG studies (two for MWCNTs and two for ZnO) were reported to assess the skin corrosion potential of MNMs. In addition to the Testing Programme, one study has been reported that applied the reconstructed skin model to test ZnO, TiO₂, and a mixture of ZnO and TiO₂ MNMs. However, as in case of TG 428 some critical factors for the evaluation of MNMs induced skin corrosion were not addressed in detail and might need to be further investigated. These include the optimum exposure duration and time of evaluation, choice of the exposure concentrations (i.e. stable dispersions without material aggregation should be used), evaluation of the cellular uptake. Although the studies did not report any apparent limitations of the application of TG 431 for testing MNMs, further studies are needed to assess the applicability of TG 431 to test MNMs.

- Applicability of TG 437 (Bovine Corneal Opacity and Permeability Test Method) should be further discussed. Under the Testing Programme, one TG study was reported to assess the potential of ZnO MNMs to cause eye irritation and damage. In addition to the Testing Programme, one study has been reported that applied TG 437 to test sixteen OECD representative MNMs. Although the studies did not report any apparent limitations of the application of TG 437 for testing MNMs, further studies that include thorough characterization of MNMs as tested in the system are needed to investigate potential interferences of the MNMs with the assay (SCCS (Scientific Committee on Consumer Safety), 2012_[20]). Literature findings indicate that nanomaterials can aggregate/agglomerate in the suspension or can absorb the dispersant and the dye, causing possible artefacts. Also, some nanomaterials present in opacity measurements may affect the result, and these should be avoided to allow consistent interpretation of.
- TG 471 (Bacterial reverse mutation test) may be amended with the acknowledgement that it is not applicable for most types of MNMs (no uptake into the bacteria). This is in line with the report from the OECD Expert Meeting on ‘Genotoxicity of Manufactured Nanomaterials [ENV/JM/MONO(2014)34] (OECD, 2014_[22]) where experts concluded that Ames test (TG 471) is not a recommended test method for the investigation of the genotoxicity of MNMs. The TG programme should consider modifying the applicability domain within this guideline accordingly.
- Modification of TG 487 (*In vitro* micronucleus assay) should be considered to include specific recommendations regarding addition of cytochalasin B when testing MNMs and the verification of intracellular uptake of MNMs. This was also one of the issues discussed in detail at the OECD expert meeting on Genotoxicity of Manufactured Nanomaterials [ENV/JM/MONO(2014)34] (OECD, 2014_[22]). The JRC is currently leading the development of an OECD Guidance Document that will support implementation of the existing genotoxicity OECD TGs on *in vitro* Mammalian Cell Based Genotoxicity when testing MNMs.
- Since several genotoxicity assays were applied in the Testing Programme, it might be most efficient to consult the expert group on genotoxicity to prioritize the assays for further harmonisation. Examples of methods that may be prioritized

include the micronucleus test (TG 487) and the *in vitro* mammalian cell gene mutation test (TG 476). The *in vitro* mammalian cell gene mutation tests is considered as an alternative for the bacterial reverse mutation test (TG 471), as reports have not yet identified any specific limitations when testing MNMs using TG 476 [ENV/JM/MONO(2014)34] (OECD, 2014_[22]).

- In addition to the work that is currently underway at the OECD WPMN and ISO TC 229 to develop standards and guidance for some cytotoxicity assays, the Testing Programme should consider evaluation and further development of the assays that are routinely used to test MNMs. For instance, the assays used to determine the potential of MNMs to cause oxidative stress and those used to assess endpoints specific to immunological implications of exposure to MNMs.

5. ABBREVIATIONS

A549:	Human adenocarcinoma alveolar basal epithelial cell line
Ag:	Silver
ATP:	Adenosine triphosphate
Au:	Gold
BEAS-2B:	Human bronchial epithelial cell line
BrDU:	5-bromo-2-deoxyuridine
BSA:	Bovine serum albumin
CHO:	Chinese hamster ovary cells
C60:	Fullerene
CeO ₂ :	Cerium dioxide
CNT:	Carbon nanotubes
DMSO:	Dimethylsulfoxide
DSBs:	Double-strand breaks
ELISA:	Enzyme-linked immunosorbent assay
EM:	Electron microscope
FACS:	Fluorescence-activated cell sorting
FISH:	Fluorescence <i>in situ</i> hybridization
GD:	Guidance document
GLP:	Good Laboratory Practice
GN:	Gold nanorods
16-HBE:	Bronchial epithelial cell line
HDMEC:	Human dermal fibroblasts
HPRT:	Hypoxanthine-guanine phosphoribosyl transferase
ICP-MS:	Inductively coupled plasma mass spectrometry
JRC:	Joint Research Centre
LDH:	Lactate dehydrogenase
MNM:	Manufactured nanomaterial

MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MWCNTs:	Multi-walled carbon nanotubes
NHEK:	Normal Human Epidermal Keratinocytes
NP:	Nanoparticle
OECD:	Organization for Economic Cooperation and Development
O/W:	Oil-in-water
SEM:	Scanning electron microscope
SiO ₂ :	Silicon dioxide
SWCNTs:	Single-walled carbon nanotubes
TG:	Test Guideline
TEM:	Transmission electron microscope
TiO ₂ :	Titanium dioxide
TOF-SIMS:	Time-of-Flight Secondary Ion Mass Spectrometry
UVB:	Ultraviolet-B radiation
V79:	Chinese hamster cells
WST:	Water-Soluble Tetrazolium salt
XPRT:	Xanthine-Guanine Phosphoribosyl Transferase
XTT:	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
ZnO:	Zinc oxide

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Annex A. Publications list

List of publications for where *in vitro* genotoxicity assays were used to test the nanomaterials (2010-2016)

PubMed Search Keywords:

- AMES test nanomaterials genotoxicity
- Chromosomal aberration in vitro nanomaterials
- HPRT genotoxicity nanomaterials
- Micronucleus in vitro genotoxicity nanomaterials
- Comet assay in vitro genotoxicity nanomaterials
- H2AX genotoxicity nanomaterials

Bacterial reverse mutation test (AMES test)

Butler KS, Peeler DJ, Casey BJ, Dair BJ, Elespuru RK. Silver nanoparticles: correlating nanoparticle size and cellular uptake with genotoxicity. (2015) *Mutagenesis* 30(4):577-91.

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In vitro mammalian chromosomal aberration test

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In vitro mammalian cell gene mutation assay using hpert gene

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In vitro micronucleus test

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