NANOGENOTOX

Facilitating the safety evaluation of manufactured nanomaterials by characterising their potential genotoxic hazard

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SUMMARY

CONTRIBUTING TO INCREASING THE SAFE USE OF MANUFACTURED NANOMATERIALS IN THE EU

Nanotechnology is a highly strategic industrial and economic sector showing enormous potential benefits for many societal and environmental domains. Human exposure to manufactured nanomaterials (MNs) present in consumer products may occur during several phases of their life cycle, from synthesis, production and inclusion in the products to the release of MNs into the environment.

The lack of scientific knowledge and the absence of evidence demonstrating the safety of some nanomaterials make regulation a challenge. In 2009 the Executive Agency for Health and Consumers (EAHC) awarded a grant through the second programme of Community action in the field of health (2008-2013) for a Joint Action (JA) on the "Safety of nanomaterials".

Towards a method for detecting the potential genotoxicity of nanomaterials

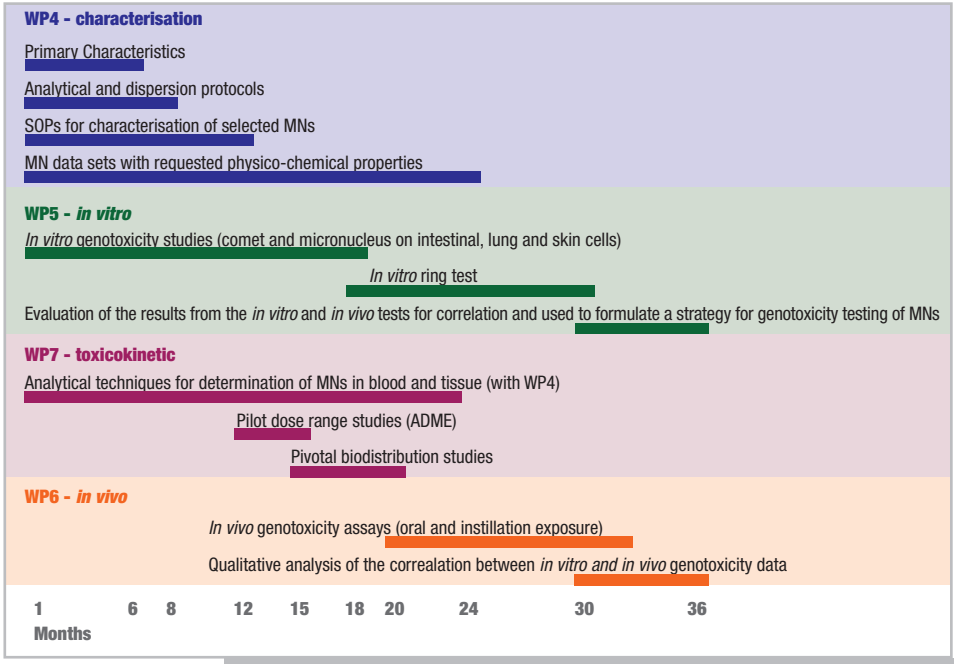
The **NANOGENOTOX** Joint Action started in March 2010 for a period of 3 years and had a total budget of over 6.2 million euros, 46% co-funded by the European Commission's Health Programme. It was coordinated by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES). Sixteen associated partners from 11 member states and 13 collaborating partners came together to pool their expertise and competences.

Associated Partners of the NANOGENOTOX Joint Action

The objective of the Joint Action (JA) was to work together towards establishing a robust (specific and sensitive) methodology to assess the potential genotoxicity (i.e. capacity to induce DNA damage) of MNs and to generate data on the genotoxic effect of certain commonly used MNs materials.

WORKING TOGETHER TOWARDS A METHOD FOR DETECTING THE GENOTOXICITY OF MANUFACTURED NANOMATERIALS

The strategy applied to reach the aim of the JA included a state-of-the-art assessment in order to identify gaps and fill these gaps, as far as possible, through testing a selected group of MNs and therefore providing generic Standard Operating Procedures (SOPs) and protocols.
The work plan consisted of four scientific work packages (WPs) and three transversal WPs devoted to the coordination, dissemination and scientific evaluation of the JA.



Forecast work plan of the NANOGENOTOX Joint Action

Fifteen MNs commercially available or soon to be on the market were kindly provided by the Joint Research Centre (from their repository) and the NRCWE: 4 Synthetic Amorphous Silica (SAS), 5 titanium dioxides (TiO_2), and 6 carbon nanotubes (CNTs) [Table 1]. They were fully characterised and then tested with standard *in vitro* genotoxicity assays completed with specific tests. Using the results, a ring test (among participating Member States' laboratories) for the relevant assays was performed to determine the reproducibility of the method. *In vivo* assays were conducted to characterise the toxicokinetics of selected MNs and to validate the *in vitro* genotoxicity data.

All the results of the project will be shared with the OECD's Working Party on Manufactured Nanomaterials' (WPMN) sponsorship programme for the testing of MNs. Synergy was also developed with other European and international activities like ISO TC229 and FP7 funded projects and networks (ENPRA, NanoSafetyCluster, Nanodevice, Q-nano etc.).

Interactions between NANOGENOTOX and other EU projects

	Ref.	Type	Major use	Tested in WP
TiO_2	NM-100	Anatase	paper loadings, rubber, cosmetics, adhesives, low cost interior paints	WP4, WP7
	NM-101	Anatase	semiconductor catalyst for use in photocatalytic processes	WP4, WP7
	NM-102	Anatase	photocatalytic	WP4, WP5 (Round Robin), WP6, WP7
	NM-103	Rutile	cosmetics (sun care, colour), pharmaceuticals, food	WP4, WP5, WP6, WP7
	NM-104	Rutile		WP4, WP5, WP6, WP7
	NM-105	Anatase/rutile	catalysis, heat stabilizer	WP4, WP5, WP6, WP7
SAS	NM-200	Precipitated	food processing	WP4, WP5, WP6, WP7
	NM-201	Precipitated	reinforcement, mechanical and optical properties and process	WP4, WP5, WP6
	NM-202	Pyrolise	inks, adhesives, cosmetics, reinforcement, powder process, food, pharmaceuticals	WP4, WP5, WP6
	NM-203	Pyrolise	food, cosmetics, pharma-ceuticals, reinforcement	WP4, WP5 (Round Robin), WP6, WP7
CNT	NM-400	MWCNT Catalytic Chemical Vapour Deposition	structural composite and energy applications	WP4, WP5, WP6, WP7
	NM-401			WP4, WP5, WP6, WP7
	NM-402			WP4, WP5, WP6, WP7
	NM-403			WP4, WP5 (Round Robin)
	NRCWE-006		lithium/ion battery	WP4, WP5, WP6, WP7
	NRCWE-007		structural composite	WP4, WP5

Table 1 – Manufactured nanomaterials selected for the JA

GOOD PRACTICE FOR THE IMPLEMENTATION OF A JOINT ACTION

Efficient project management refers to the planning and organisation of the activities to ensure that the Consortium delivers the expected outputs in due time and budget. This requires in particular a sufficient level of organisational capacity by the participants, a strong partnership coupled with the implementation of a targeted communication strategy and a systematic evaluation of the activities.
The three horizontal Work Packages (Coordination, Dissemination and Evaluation) implemented a set of tools to improve the chances of success as well as a series of processes to monitor time, costs, quality and scope of the project.

Coordination

As Coordinator, ANSES was in charge of the continuous and consistent operation of the JA and the direct link between the Partners and the EAHC. The Coordination Team put in place by ANSES monitored the scientific, financial and knowledge management contractual obligations including reporting to the EAHC and budget consumption.
Different levels of project meetings were set up: at *the Executive level* monthly Coordination Team meetings within ANSES to closely follow up the overall scientific progress, time schedule and budget; at the *Strategic* level Steering Committee meetings, at least every 3 months, to oversee the work and progress of each individual WP, especially milestones with regards to objectives and timetable. The ultimate decision body, the General Assembly which brought together all the partners of the Action plus the collaborating partners met twice a year.

5th General Assembly meeting at INSA (Portugal). Credit INSA.

The rules of the Consortium were precisely defined in a Consortium Agreement established by the Coordinator in order to secure the partnership by a formally signed agreement contractualising the cooperation, organising the legal and operational framework of the Joint Action, the access rights and defining the rights and obligations of the partners.

Reaching the stakeholders

Dissemination is the process of engaging with, and making the results and deliverables available to, the stakeholders and a wider audience. The key elements for appropriate targeting are to carry out a stakeholder analysis, ensuing consultations and adapting the dissemination and sustainability strategy to the expectations of the stakeholders.

Stakeholders selected for the consultation had to be involved at an EU (or international) level, recognised in their domain and exercising a certain influence, recognised for their ability to relay information to a wider audience, possess technical and scientific knowledge regarding nanotoxicology and be willing to engage in technical discussions about nanotoxicology.

Five categories of stakeholders were identified:

- EU risk assessors and policy-makers,
- members of the scientific community,
- professional federations representing companies,
- non-governmental organisations (NGOs),
- trade unions.

The stakeholders' input is invaluable in helping to identify the concerns and needs of the various groups of stakeholders, for example, the needs of industry regarding to safe design or for the preliminary testing of a nanomaterial that is still under development before investing important efforts and money.

Although the objective was not to directly look into risk assessment and risk management concerns, policy-makers were involved in at least two ways: through certain partners which are risk assessment institutes with strong links, as knowledge brokers, to ministries, and directly as ministries of several Member States were collaborating partners.

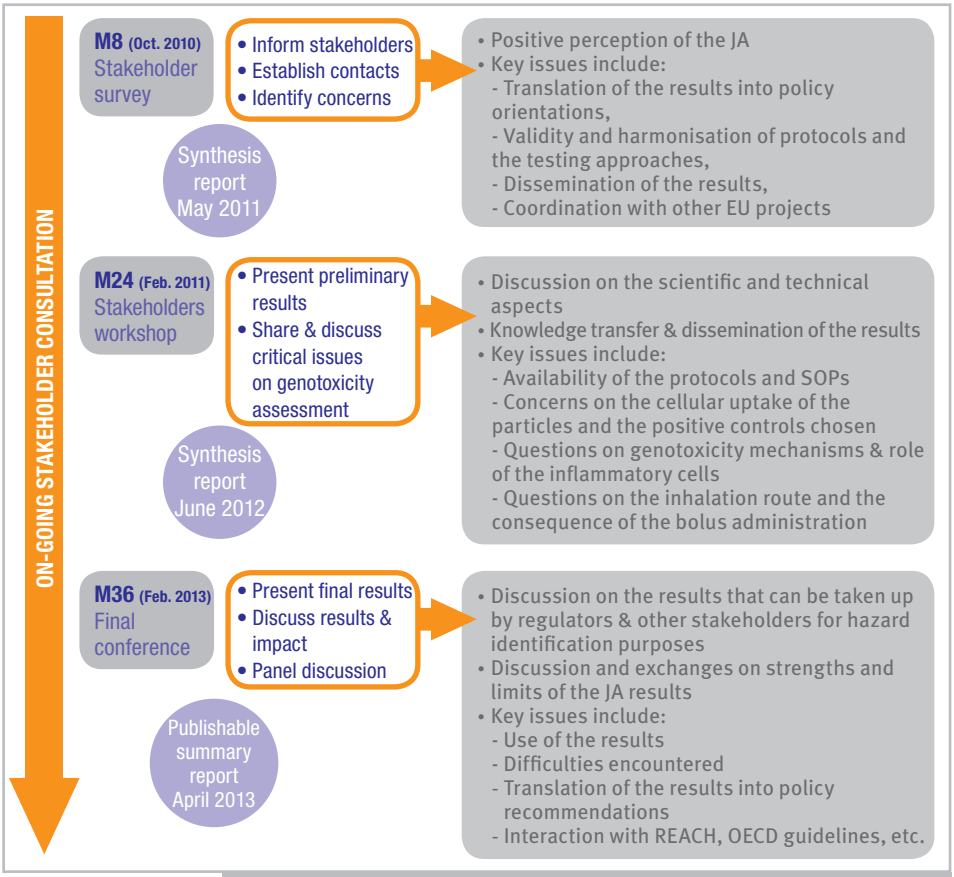


Figure 1 – Stakeholders consultation process

The final conference of NANOGENOTOX took place on Friday 22 February 2013 and was hosted by the French Ministry of Social Affairs and Health, in Paris, France. It gathered around 200 participants from all over Europe and beyond, including partners of the Joint Action.

The results of each WP were presented and discussed in the morning sessions. In the afternoon, a presentation summarising the considerations to achieve a robust method for testing the genotoxicity of MNs was made, followed by discussions with the audience. Particular efforts were made during the panel discussion and in the conclusions and perspectives by policy-makers to examine how this method can be taken up and followed upon within REACH or other regulatory mechanisms.

As policy-makers, the OECD WPMN and the EC DG SANCO representatives confirmed that the results and lessons coming out from NANOGENOTOX can be built upon for risk assessment and risk management purposes. Existing frameworks and guidelines are applicable for nanomaterials but some particular test guidelines have to be examined closely and may need to be updated or replaced. The concerns identified in the NANOGENOTOX findings are shared by the EC Scientific Committee on Consumer Safety (SCCS).

Final conference, 22 February 2013, Paris (France). Credit ANSES.

Like most collaborative projects, NANOGENOTOX developed classical tools for raising awareness: a web site (www.nanogenotox.eu) and a leaflet, giving an identity and explaining the objectives and planned activities of the JA. Newsletters focusing on the results were published every 6 months. Partners participated in national and international conferences to present their results, and agreements on publications have been reached in each scientific WP.

Evaluation of the Joint Action

Evaluation is an important process in order to assess if the project objectives have been achieved and whether the needs of the stakeholders have been met. Therefore, the quality of the work and the ability of the JA to respond to the requirements of the EU Health Programme were systematically evaluated during the course of the JA.

An Internal Evaluation Team, composed of one representative from each Work Package, monitored and analysed the quantitative (number of

datasets) and qualitative (robustness and reliability of the tests) specific objective indicators according to an approved evaluation plan defined early in the project by the Evaluation Work Package (WP3).
Following the Evaluation plan which included defined templates, questionnaires and indicators as well as timelines, confidential "cruise mode" evaluation reports were completed every 6 months reviewing the JA data generation and knowledge sharing actions. In these reports, recommendations were made by the Evaluation Team in order to keep up with the work plan and to take corrective actions.
In addition, in cooperation with the Coordination Team, an external academic reviewer panel was created to evaluate the Joint Action's deliverables and to respond to any specific issues that the internal Evaluation Team might have. The external reviewers were selected for their excellent scientific record in the respective fields of expertise. They participated in a final review meeting in Berlin in November 2012 with the Internal Evaluation Team and the work package leaders to discuss in depth the preliminary results as well as the Deliverables (as available at that point). A number of them participated in the Final conference to share with the conference participants their evaluation of the JA's scientific outputs.

3rd GA meeting at NRCWE (Denmark), October 2011. Credit ANSES.

CHARACTERISING MANUFACTURED NANOMATERIALS AND EXPOSURE MEDIA

Objectives

Verification and detailed information on test materials are essential for proper interpretation of experimental results. Reliable data on physico-chemical characteristics of MNs become especially crucial when such results are applied in furthering a new understanding of nanomaterial toxicity and in producing advice for regulatory use. Therefore, reliable high-quality methods for characterisation of the materials as such, but also their exposure characteristics in both air and liquid dispersions are highly necessary and currently under continuous development. In response to the requirements in the NANOGENOTOX project, the main objectives of WP4 were to:

- test and develop suitable methods and Standard Operating Procedures (SOPs) for analysis and characterisation of MNs and dispersions thereof,
- determine the intrinsic characteristics of MNs selected for toxicological studies,
- test the homogeneity of the MN batches distributed,
- develop, test and verify highly suitable MN dispersion protocols to be used in toxicity testing.

Materials

All selected MNs were characterised and the primary characterisation data constituted part of the selection criteria for the toxicological studies. Table 2 shows the complete material list and selected material characteristics obtained in the JA.

Methods and SOP developments

Numerous different methods and procedures could be applied for characterisation of the MNs, their reactivity, exposure characteristics and emission potential. Therefore, the WP had to focus on a number of key characteristics to be studied. These characteristics included the primary and secondary (aggregate) average particle sizes (or distribution), morphologies of particles and fibers, their atomic structure, chemical compositions, contaminants, catalysts and associated organic matter, as well as primary surface charge given by the zeta-potential as a function of pH. Another task was aimed at characterising the biologically relevant hydrochemical reactivity (OH radical formation capacity, causticity, O_2 or redox-activity) and short-term solubility of the individual MNs. Finally,

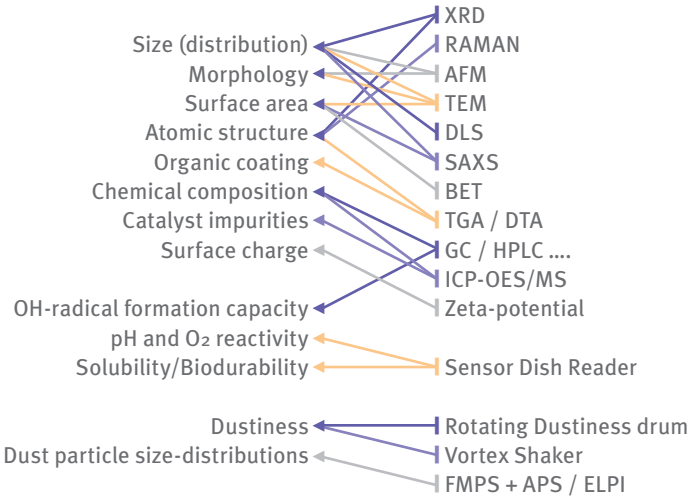
	Powder						Batch dispersion for toxicology	
Sample	Phase	Average XRD crystallite size	Average TEM particle size	Average BET & SAXS SSA[£]	TGA mass-loss	Main elemental impurities	Average SAXS aggregate size	Average DLS Zeta-size
NM-100	anatase	56.7 - >100 nm	110 nm	9 m^2/g	nd	K,P	NA	215 nm
NM-101	anatase	7 nm	6 nm	316 m^2/g	8 wt%[€]	Al,Na,P,S,Zr	NA	483 nm
NM-102	anatase	21 nm	22 nm	78 m^2/g	nd	S	560 nm	545 nm
NM-103	rutile	23 nm	25 nm	51 m^2/g	2 wt%[€]	Al,Si,Na,S	140 nm	194 nm
NM-104	rutile	23 nm	25 nm	56 m^2/g	2 wt%[€]	Al,Si,Ca,Na,S	160 nm	234 nm
NM-105	anatase rutile	23 nm 60 nm	24 nm 15 nm	46 m^2/g	nd	nd	130 nm	155 nm
NM-200	SAS	NA	18 nm	189 m^2/g	3 wt%[€]	Na,Al	440 nm	185 nm
NM-201	SAS	NA	18 nm	140 m^2/g	3 wt%[€]	Na,Al	180 nm	176 nm
NM-202	SAS	NA	18 nm	204 m^2/g	nd	Ca,Al	100 nm	134 nm
NM-203	SAS	NA	25 nm	204 m^2/g	nd	Al	NA*	127 nm
NM-204	SAS	NA	16 nm	137 m^2/g	<1 wt%[€]	Na,Al	NA	174 nm
NM-400	MWCNT	NA	d=14nm; L<1µm	254 m^2/g	84 wt%	Al,Fe,Co,Zn,Na	NA	55 nm[$]
NM-401	MWCNT	NA	d=64nm; L <5µm	18 m^2/g	82 wt%	Fe,Zn,Na	NA	710 nm[$]
NM-402	MWCNT	NA	d=13nm;L<5µm	226 m^2/g	89 wt%	Fe,Al,Na	NA	NA*
NM-403	MWCNT	NA	d=12nm;L<0.5µm	135 m^2/g	97 wt%	Co,Mn,Mg,Al,Na	NA	NA*
NRCWE-006	MWCNT	NA	d=74nm;L<10µm	26 m^2/g	82 wt%	Co,Fe,Mg,Al,Na	NA	682 nm[$]
NRCWE-007	MWCNT	NA	d=17nm;L<0.5µm	96 m^2/g	94 wt%	Ni,Fe,Cr,Co,Na	NA	223 nm[$]

NA: Not applicable/Not available; [€]ascribed to organic coating/functionalisation; [£]The average of one BET and one SAXS determination. [$]Note that DLS size of CNT is an apparent number; * Not sizeable; nd: not detected

Table 2 – Summary of samples and average data on key analytical results

the size-distributions and potential of the powder MNs to release dust during handling was assessed by dustiness testing using two fundamentally different approaches. A complete list of the specific characterisation items and applied methods addressed in WP4 are shown below.

Regarding the methods, several international (e.g. ISO, CEN and OECD) particle and material characterisation SOPs may in principle be applicable to MNs. However, in many cases, method validation on different materials, adjustments for nanomaterials or final consensus on procedures has yet to be made. All participants in WP4 had previous experience in MN characterisation, and some had already been in the process of improving or establishing new procedures for characterisation of MNs.



Abbreviations: XRD (X-ray diffraction); Raman (Raman Spectroscopy); TEM (Transmission Electron Microscopy); AFM (Atomic Force Microscopy); DLS (Dynamic Light Scattering); SAXS and uSAXS (Small Angle X-ray Scattering and ultrafine SAXS); BET (Brünauer, Emmett and Teller gas adsorption); TGA (Thermogravimetric analysis); DTA (Differential Thermal Analysis); GC (Gas-Chromatography); HPLC (High-Performance Liquid Chromatography); ICP-MS (Inductively Coupled Plasma Mass Spectrometry); FMPS (Fast Mobility Particle Sizer); APS (Aerodynamic Particle Sizer); ELPI (Electrical Low-Pressure Impactor).

Figure 2 – Summary list of characteristics and analytical methods applied in NANOGENOTOX

Therefore, it was decided to follow or further develop internal best procedures in the characterisation work and thereby propose first drafts of SOPs to be further developed or used for validation in other international collaborations. It is already planned that some of the NANOGENOTOX procedures will be further investigated in the EU FP7 co-funded project NANoREG "Common European approach to the regulatory testing of nanomaterials", which specifically aims at developing SOPs for regulatory purposes. In the following section, the conclusions of the primary NANOGENOTOX characterisation work, which includes the analysis of the primary physico-chemical characteristics of the test materials and development of the generic NANOGENOTOX dispersion protocol, will be discussed.

The Primary MN characteristics

Particle size

The primary particle size (-distribution) is the key criteria for defining a MN, in accordance with the proposed EC nanomaterial definition, and it may be analysed by a number of different methods. In NANOGENOTOX, XRD, TEM, AFM, DLS and SAXS were used as complementary methods for describing the particle size.

XRD is only applicable to crystalline materials and therefore has limited general use in size-analyses. However, XRD is an excellent method for analyses of the mineralogy and significant crystalline impurities in bulk powder samples. Advanced mathematical analyses of XRD data enable quantification of the average crystallite size, preferred orientations, in a nanocrystalline material as well as estimates of the proportion between phases in a sample. However, the project data also indicate that the size and proportions obtained may be dependent on both instruments and the data-analyses method applied in the calculations.

TEM and AFM are generally considered to be among the most precise typical methods for measurements of the particle size-dimensions in the nanoscale. In TEM, the particle size is measured on highly magnified digital images and it may be completed following automated, semi-automated and manual methods. Some of the challenges in TEM size-distribution measurements include reliable sample preparation that captures and maintains the real particle size-distribution of the samples, as well as the very large span in size dimensions observed for some MNs. The latter is evident in the case of MWCNT, where the thin diameter of the CNT may range from ca. 1 nm to more than 100 nm while the lengths may vary from nm-scale to several tens of µm's. In addition MWCNTs may be very flexible causing complex structures and agglomerates and therefore difficulties in sizing the tube length [Figure 3]. Another issue related to morphology is primary particle size-distribution measurements of dispersed and non aggregated particles to nanostructured and fused particles or structures such as the synthetic amorphous silica [Figure 3].

For sample preparation, the study concluded that powder samples should be pre-sonicated in a suitable dispersion medium to disperse large agglomerates and aggregates before adding them to TEM-grids. The exact medium, the sonication power and duration must be optimised for each specific MN. For example, pure double-distilled water is sufficient to disperse most SAS, whereas acidified water may be used for TiO_2. For CNT addition of bovine serum albumin was proven to be applicable. However, other alternatives certainly exist.

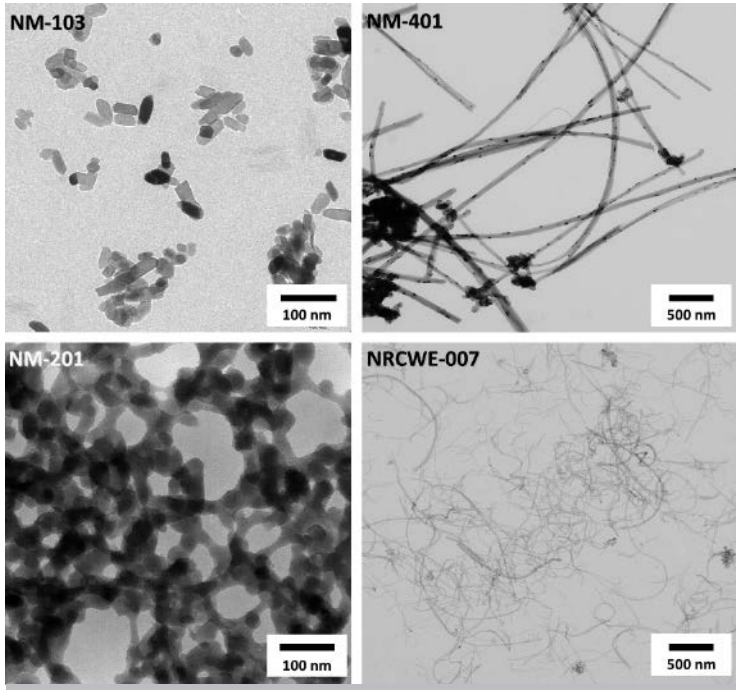


Figure 3 – Examples of different states of particles and particle morphologies of MNs. NM-103 is a coated TiO_2, NM-201 is SAS, NM-401 is a wide-diameter MWCNT with presence catalyst particles (dark spots) at the rim of the CNT. NRCWE-007 is a flexible and bendable MWCNT with highly variable diameters. Credit IMC-BAS, CODA-CERVA.

Regarding data acquisition, automated analyses are preferred. It allows immediate measurement of multiple parameters and reduces operator-induced bias and assures a statistically relevant number of measurements. Key data appear to be the mean particle diameter and the Feret mean, which are the results of multiple diameter measurements on each particle. The equivalent circular diameter, which is calculated from the projected surface area, is another useful parameter. For categorisation of a material as a MN or a bulk material, according to the EU proposal for a definition of a nanomaterial, the percentage of particles and aggregates smaller than 100 nm can be calculated from the number-based distribution of Feret min, which is the estimate for the minimal size for each particle. For TiO_2 and SAS, the established procedures seem to work well. For MWCNT, issues remain regarding reliable automated analysis and there are concerns regarding length determinations. In all cases lower and upper boundaries for sizing should be identified in future work.

Analysis of particle size in liquid dispersions

SAXS and DLS are two very different techniques and were used to measure the size-distribution of particles in liquid suspensions. Whereas the DLS measures the particles by their Brownian motion, SAXS measures the particles according to mathematical treatment of scattered X-ray data. Both methods may be applicable for measurement of aggregate size-distributions after appropriate dispersion in a liquid. However, SAXS can also be used to derive information on the aggregate/agglomerate structure, the average primary particle size and the average shape factor and can also be used on dry powders. Both methods were assumed to be applicable both for SAS and TiO_2 samples, but uncertainties are still raised regarding the applicability for measurements on CNT. Comparison between aggregate sizes determined in batch dispersion media prepared according to the generic NANOGENOTOX dispersion protocol for toxicity testing suggests that SAXS gives smaller average aggregate sizes than DLS, when the intensity-derived hydrodynamic zeta-average values are used from the DLS.

Analyses of particle size in dustiness testing

A last method for investigating the particle size-distributions of the powder samples was applied as part of dustiness testing. We measured the dustiness and dust size-distributions generated by two different methods: a downscaled standard EN15051 rotating drum and a Vortex shaker. Both methods are candidates for a new nanopowder dustiness standard in EC Mandate 461 for standardisation activities regarding nanotechnologies and nanomaterials.

Sizing with a Fast Mobility Particle Sizer plus an Aerodynamic Particle Sizer (FMPS+APS) in the rotating drum test and an Electrical Low-Pressure Impactor (ELPI) in the Vortex shaker tests showed that the powder dusts all had bi- to multimodal number size-distributions. The dust particle size-ranges were very wide ranging from less than 100 nm and into the µm size-range. However, it was also evident that either the two types of tests produce dusts with different size-distributions or the different instruments give different method-dependent size-distributions.

The FMPS+APS measurements of TiO_2 and SAS dusts showed peak number concentrations for particle sizes around 200 and 300 nm, respectively, but size-modes were also observed around 40-60 nm for some powders and in the µm-range for all powders. ELPI measurements showed more variation in the number peak-size where both TiO_2 and SAS usually had a primary or secondary peak-size around or below the 100 nm size

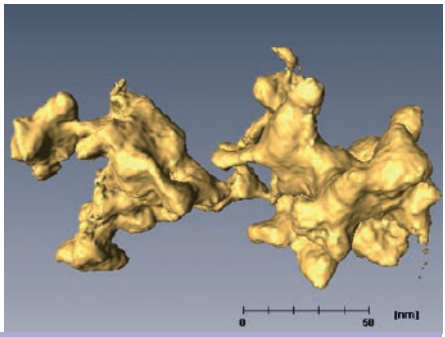
range. In all cases the majority of dusts from TiO_2 and SAS were released as aggregates and/or agglomerates. The MWCNT (NM-400, 401, 402, and 403) were only analysed using the Vortex shaker method. The experiments revealed that both free and agglomerated/aggregated CNT "fibers" were released during this agitation procedure. The fraction of dust particles smaller than 100 nm was very significant in tests of NM-400, NM-402 and NM-403. In NM-401, the peak size in particle number was located between 200 and 300 nm.

Specific surface area (SSA)

Specific surface area analyses were performed by BET and SAXS. Experimentally, SSA was also determined by TEM tomography on single samples. BET is surface area measurement based on quantification of the amount of nitrogen gas that was adsorbed to the powders, whereas SAXS relies on mathematical analyses of the X-ray scattering signal from the particle surfaces in the powder sample. Determination of SSA or VSSA (Volume-Specific Surface Area) by TEM tomography is based on 3D morphological analysis of each powder particle in a sample.

The results showed a wide range in the specific surface areas of the MNs analyzed with BET and there was a general linear correlation between data obtained by BET and data obtained by SAXS. Above ca. 130 m^2/g the SAXS data appeared to underscore the specific surface area determined by BET. However, more samples with a wide range in SSA are required to fully assess the comparability between these two methods. The tomographic approach also appears to give reliable values, but particle-specific data have not been obtained on standards or by other methods in this project. In addition the technology is not yet ready for high-throughput analyses on all types of materials therefore a final conclusion cannot be made on the applicability of this procedure.

Tecnai spirit transmission electron microscope with biotwin lens configuration operating at 120 kV. Credit CODA-CERVA.



Transmission electron tomographic reconstruction of aggregated SAS nanoparticles. Bar is 50 nm. Credit CODA-CERVA.

Chemical compositions, impurities and coatings

The elemental chemical composition of the nanomaterials and associated organic compounds are obviously of high interest, beyond the identification and categorisation of the MN. The presence of inorganic minor elements, either as substitutions of constituent elements in the MN atomic structure or due to an inorganic coating, may change the toxicological effect of a MN. Similar effects have been observed due to organic coatings and functionalisations of MN as well due to the presence of different catalyst materials e.g., in MWCNT.

Clearly different techniques are required to identify and quantify inorganic elements and associated organic compounds as contaminants, coatings and functionalisations. Even though extensive development of chemical analysis was not part of this project, procedures were refined to improve extraction for elemental analysis of inorganic MWCNT catalysts by ICP. In addition, a procedure was established in synergy with two EU FP7 projects, NANODEVICE and ENPRA, using thermogravimetric analysis to identify whether a MN may be constituted of or associated with organic compounds. Mass losses due to compounds evaporating at temperatures greater than 105°C were discriminated from mass-losses occurring at lower temperatures, which could be due to adsorbed water, for example. In the current work it was decided that any inorganic material with a total mass-loss greater than 1 wt% during combustion in air would be subject to subsequent organic chemical analysis. For MWCNT, the residual mass after combustion was used to determine the amount of inorganic catalyst material in the samples.
Inorganic chemical analysis by EDS showed the presence of ca. 4.5 wt% Al and 0.7 wt% Si in NM-103 and NM-104 (TiO_2) as well as about 0.5 wt%

Fe in NM-400. Al was known to be present in inorganic coatings in NM-103 and NM-104. All other TiO_2 samples contained less than 0.2 wt% Si. Other minor impurities were due to salts. NM-101, NM-104 and NM-104 had a TGA mass-loss of 2 to 8 wt% that could be ascribed to organic coatings.

EDS and ICP analysis both showed that the SAS MNs all contained minor amounts of Al. Other minor impurities were due to salts. TGA showed a mass-loss of 1 to 3 wt% in NM-200, NM-201 and NM-204.

Quantitative elemental analysis of MWCNT was found to be a greater challenge than may be generally anticipated. There was poor agreement between the results coming from different laboratories. None of the elemental analysis reached the 3 to 18 wt% impurities indicated by TGA. Combining results from EDS-analysis and different ICP methods as well as XRD on residuals after combustion confirmed the major impurities to be various combinations of transition metal catalysts. However, a significant fraction may also sometimes be ascribed to salts, which are thought to be residuals after purification of the CNT.

Dispersion of MNs for toxicity testing

Focus on protocol for producing suitable manufactured nanomaterial exposure media

As a major and very early deliverable, WP4 was requested to develop Standard Operating Procedures (SOPs) for preparing MN batch dispersions suitable for application in *in vitro* and *in vivo* toxicity testing. It was agreed that WP4 would produce a generic dispersion protocol aiming to:

1. use the biologically relevant serum albumin for particle stabilisation (surfactant),
2. reduce the albumin concentrations as much as possible to limit potential unwanted toxicological side-effects and
3. adopt the batch dispersion MN concentration and probe sonication conditions already established in the EU FP7 project, ENPRA.

Based on these boundary requirements, a series of range-finding tests were conducted to identify applicable BSA (Bovine Serum Albumin) concentrations to achieve relatively stable MN dispersions, procedures to also enable dispersion of hydrophobic MNs, detailed adjustments and harmonisation of the sonication vials, preparation

volumes, and sonication conditions, including selection of proper immersion depth of the sonicator probe, and adjustments of sonication times for sonicators with different sonication energies and amplitudes.
The work finally resulted in a common generic dispersion protocol using a 0.05% w/v sterile-filtered BSA-water solution as dispersion medium for 2.56 mg MN per ml, total batch dispersion volume 6-10 ml, generic pre-wetting of the MN powder with 0.5% (v/v) ethanol to also enable dispersion of hydrophobic MN, and probe-sonication for 16 min at 400W and 10% probe-amplitude while keeping the sample vial cooled in an ice-water bath. Sonicators with higher energy output should apply reduced sonication times (e.g., 12 min at 500 W and 20% amplitude). The detailed dispersion protocol is available on the JA web site.
It is important to note that NANOGENOTOX protocol is a procedure which is generally applicable to all powder MN. It will not always, and probably rarely, disperse MNs into their primary particles or aggregates. However, in most cases, the protocol produced metastable dispersions with derived DLS number peak-sizes within the lower and upper TEM size-range found for the MN and less than 10% sedimentation within the first 1 hour. Introduction of mandatory brief vortex shaking of the batch dispersions immediately before use ensured re-establishment of the original characteristics of the batch dispersions. It should be noted that the MN dispersions should always be used immediately after preparation in order to minimise potential artefacts induced by particle dissolution and / or exhausted surface reactivity.

IN VITRO METHODS FOR GENOTOXICITY TESTING

Objectives

The basic questions of *in vitro* genotoxicity testing of manufactured nanomaterials (MNs) include how well *in vitro* assays can be used for revealing the genotoxic potential of MNs, which assays are suitable for this task, and which modifications are needed in the tests when MNs are studied. The main aim of WP5 was to establish robust methodology to screen *in vitro* genotoxicity of MNs in pulmonary, intestinal and dermal cell systems. The first objective was to generate *in vitro* genotoxicity data on the chosen MNs, using standard tests and modified assays utilising specific cell models. The second objective was to evaluate the robustness of the methodology. Based on *in vitro* genotoxicity test results, a round robin test was carried out, using the most promising *in vitro* assays.

Methods

Three genotoxicity endpoints were chosen for the first part of WP5: DNA damage, micronuclei formation, and mutations.

Alkaline and FpG-modified comet assays were used for assessing DNA damage. The alkaline comet assay is a simple and sensitive method for the detection of DNA strand breaks (single- and double-strand breaks) and alkali-labile sites. The FpG-modified assay allows the detection of oxidative DNA damage.

The micronucleus assay was used to detect agents that modify chromosome structure or their segregation, leading to the formation of an additional nucleus (micronucleus) during cellular division. The cytokinesis block micronucleus assay, using cytochalasin B to prevent cytokinesis, was performed in all cell lines, except 16 HBE cells where cytochalasin B was not used.

The mouse lymphoma assay was carried out to detect mutations.

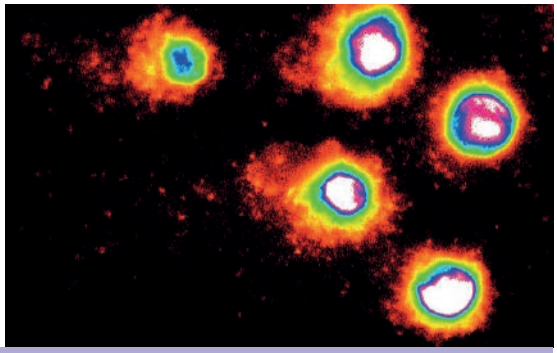
Various human cell lines of different origin were used: pulmonary (bronchial epithelial BEAS 2B and 16 HBE; alveolar A549), intestinal (Caco-2, primarily undifferentiated cells used) and epidermal (NHEK keratinocytes).

Reconstructed full-thickness skin models were applied only for testing of TiO_2 and ZnO nanoparticles. The micronucleus assay was performed also in human primary lymphocytes. The mouse lymphoma mutation assay was carried out in mouse lymphoma L5178Y TK+/- cells.

In vitro comet and micronucleus assays, complemented with the mouse lymphoma assay, were applied to all MNs assessed (except in the dermal systems, where only TiO_2 was tested) using the dispersion protocol

provided by WP4. Most series included nanosized ZnO as a nanoparticle control, in addition to assay-specific (chemical) positive controls: mitomycin C in the micronucleus assay, ethyl methane sulphonate or methyl methane sulphonate (MMS) in the comet assay, and MMS in the mouse lymphoma assay.

The protocols were harmonised, following the general principles described below. One experiment was performed per MN per genotoxicity endpoint (two experiments when time allowed it). Each treatment was conducted as duplicate cultures. No metabolic activation system was utilised. The comet assay was carried out with two treatment times, 3 h or 24 h. For the micronucleus assay, a longer-term treatment covering 1.5-2 cell cycles was used; cytochalasin B was added 6 h after the start of the treatment, in Caco-2 cells at 24 h. The treatments were performed in the same medium that was used for the culture. The doses of the MNs tested were chosen on the basis of cytotoxicity measurements using mostly cell count relative to control, relative increase in cell counts (RICC), or relative population doubling (RPD). The highest dose was either at the cytotoxicity limit of 55% +/-5% or as otherwise justified. For each nanomaterial, 4-6 doses were included in the genotoxicity assays to obtain a minimum of 3 analysable doses. In the case of MNs with low cytotoxicity, the maximum dose was derived from the WP4 dispersion protocol (256 µg/ml) or was based on technical limitations (e.g. inhibition of analysis because cells were covered with MNs). Doses were given in µg/ml and (for cells growing on surface) also in µg/cm^2. The results were related to specific surface area (if possible) and other characteristics considered important, to find out if they correlated with genotoxicity.



A fluorescence micrograph (propidium iodine staining) of human NHEK cells treated with NM-104 for 24 h, as processed for the comet assay. Damaged DNA is seen as a red «comet tail» on the left side of the cell nucleoids. Credit IMB-BAS.

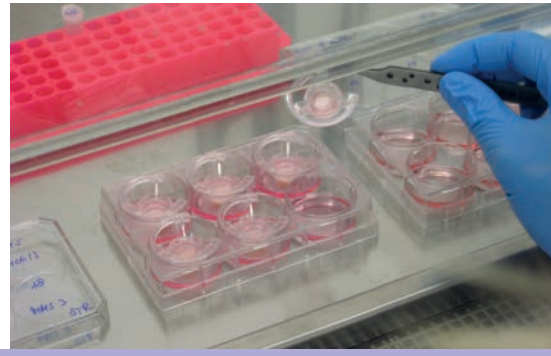
In the context of the development of experimental methodologies, a round robin study (an inter-laboratory test performed independently in 12 different laboratories), was carried out to assess the reproducibility of the genotoxicity tests. The round robin study comprised *in vitro* genotoxicity testing of one type of each family of studied MNs: TiO2 (NM-102, doses: 0, 64, 128 and 256 µg/ml), SAS (NM-203, doses: 0, 8, 32 and 64 µg/ml), and MWCNT (NM-403, doses: 0, 64, 128 and 256 µg/ml); both the cytokinesis block micronucleus assay and the alkaline comet assay were carried out. ZnO (NM-110, doses: doses used between 1.5 and 8.55 µg/ml) was included in all series, to assess its possible use as a nanoparticle positive control. The partners were divided into two groups, one group of six laboratories using bronchial human epithelial BEAS 2B cells, and the other six laboratories using human epithelial colorectal adenocarcinoma Caco-2 cells.

1st part – *in vitro* results

TiO_2

The micronucleus assay was positive[1] for each TiO2 in NHEK cells. There was also a positive finding for lymphocytes for NM-102, NM-103 and NM-104. The micronucleus assay was negative for all TiO_2 in other types of cells.

The comet assay was positive for all TiO_2 in Caco-2 cells after the 24-h treatment except for NM-104 (negative). Results of the comet assay were positive, with the 3-h or 24-h treatment for NM-102 in all cell lines except 16HBE and for NM-105 in all cell lines except BEAS 2B and 16HBE.



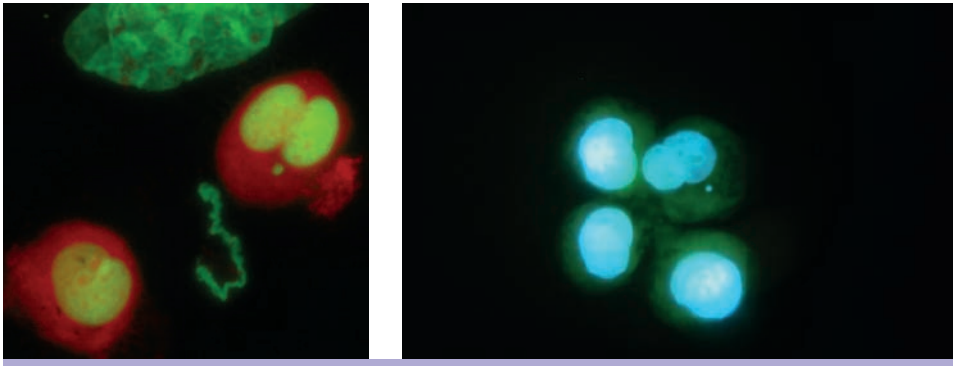
Full thickness skin models (EpiDermFTTM). Credit BfR.

1. Positive result: a statistically significant increase with ≥2 doses or a statistically significant increase at high dose and a dose-dependent increase.

The FpG-modified comet assay was positive for NM-104 and NM-105 in BEAS 2B and Caco-2 cells, and for NM-104 in A549 cells, but negative for all types of TiO_2 in 16HBE cells.
The mutation assay was negative for all forms of TiO_2 tested.
In 3D human reconstructed full thickness skin models, all TiO_2 nanomaterials (NM-102, NM-103, NM-104, and NM-105) investigated for DNA damage were negative in the comet assay. In contrast, the chemical positive control MMS consistently generated a significant increase in DNA-damage. The highest dose studied by this protocol was 246 µg/cm² skin surface which showed no interference during the analysis. Transmission electron microscopic analysis by CODA-CERVA could not identify penetration of TiO_2 through the stratum corneum of reconstructed human full thickness skin models even after 72 h exposure. This points to an undisturbed skin barrier in these 3D models and may explain the lack of positive results as compared to results obtained with NHEK cells. As TiO_2 nanoparticles showed no penetration, the *in vitro* micronucleus assay was not systematically carried out with the 3D human skin models, but a more in depth investigation was performed using the comet assay. The probability of those nanomaterials reaching dividing cells of an intact 3D skin barrier is close to zero. Furthermore, the full thickness skin models appear to show only a low cell division rate in the end-differentiated stage. Thus, it is postulated that nanomaterials with a realistic agglomerate size above 20 nm will not enter viable human skin models and consequently will not exert genotoxic effects in this test system.

SAS

All SAS nanomaterials induced micronuclei in Caco-2 cells, but when the experiment was repeated, the initial positive results could not be confirmed. NM-201 and NM-202 induced micronuclei in A549 cells. The micronucleus assay was mostly negative for all SAS in other cells.
After the 3-h treatment, the comet assay was mostly positive for the different types of SAS in BEAS 2B cells and for NM-200 in all cell lines. Positive results were also obtained in the comet assay with NM-201, NM-202 and NM-203 in A549 cells after the 3-h or 24-h treatment and with NM-203 in Caco-2 cells (both treatment times).
The mutation assay was negative for all types of SAS tested.



Fluorescence micrographs of Caco-2 cells stained with (left) acridine orange and (right) DAPI (4',6-diamidino-2-phenylindole). Both figures show a binucleate cell containing a micronucleus. Credit ANSES.

MWCNT

The micronucleus assay was mostly positive for MWCNTs in BEAS 2B, A549 and Caco-2 cells, but negative for all MWCNTs in 16HBE cells.

The comet assay (with and without FpG) and the mutation assay were negative for all MWCNTs.

		Pulmonary										
		BEAS 2B					16 HBE					
		Micronucleus[a]	Comet 3h	Comet 24h	Comet FpG 3h	Comet FpG 24h	Micronucleus[b]	Comet 3h	Comet 24h	Comet FpG 3h	Comet FpG 24h	Micronucleus[a,c]
TiO_2	NM-102	-	+	+			-	-	-			-
	NM-103	-	-	-			-	-	-			-
	NM-104	-	-	-			-	-	-			-
	NM-105	-	-	-			-	-	-			-
SAS	NM-200	-	+		(+)		-	+	-	-	-	-/-
	NM-201	-	(+)		-		-	-	-	-	-	+/+
	NM-202	-	+		+		-	-	-	-	-	+/+
	NM-203	(+)	+		+		-	-	-	-	-	-/(+)
MWCNT	NM-400	(+)	-	-	-	-	-	-	-			(+)
	NM-401	+	-	-	-	-	-	-	-			-
	NM-402	+	-	-	-	-	-	-	-			+
	NM-403	+	-	-	-	-	-	-	-			-
	NRCWE-006	+	-	-	-	-	-	-	-			+
	NRCWE-007	+	-	-	-	-	-	-	-			+

+ Positive: a statistically significant increase with ≥2 doses or a statistically significant increase at high dose and a dose-dependent increase;
(+) Equivocal: a statistically significant increase with 1 dose, no dose-dependent increase;
- Negative;
/ Used to separate outcome of two experiments.
Grey box, Not performed.

				Intestinal					Lymphatic		Dermal				
A549				Caco-2					Lymphocyte	L5178Y TK+/-	NHEK			Skin 3D	
Comet 3h	Comet 24h	Comet FpG 3h	Comet FpG 24h	Micronucleus[d]	Comet 3h	Comet 24h	Comet FpG 3h	Comet FpG 24h	Micronucleus[e]	MLA	Micronucleus[f]	Comet 3h	Comet 24h	Comet 3h	Comet 24h
+	-			-	-	+			(+)	-	+	(+)	(+)	-	-
-	-			-	-	(+)			+	-	+	(+)	(+)	-	-
-	-			-	-	-			+	-	+	(+)	(+)	-	-
+	-			-	-	+/+			-	-	+	(+)	(+)	-	-
(+)	-	-	-	+/-	+	+	(+)	+	-	-					
+	(+)	-	(+)	+/-	-	(+)	-	+	-	-					
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-	-			+	-	-	-	-	(+)	-					
-	-			(+)	-	-	-	-	+	-					
-	-			-	-	-	-	-	+	-					
-	-			+	-	-	-	-	-	-					

a Treatment for 48h, Cyt-B added at 6h.
b Treatment for 41h, no Cyt-B used.
c Treatment for 24 h (TiO2, NRCWE-006 and NRCWE-007), Cyt-B added at 6h.
d Treatment for 52 h, Cyt-B added at 24 h.
e Treatment for 30 h, Cyt-B added at 6 h.
f Treatment for 54 h, Cyt-B added at 6h

Table 3 – Outcome of the *in vitro* genotoxicity assays in various cell systems (first part of WP5)

2nd part – Round robin test

NM-102 (TiO_2)

In BEAS 2B cells, the outcome of the comet assay with NM-102 was almost unanimously positive (five out of six laboratories), in accordance with the outcome of the 1st part of WP5. The result of the micronucleus assay in BEAS 2B cells was negative in four laboratories (in agreement with the 1st part), positive in one laboratory and equivocal in another laboratory.
In Caco-2 cells, the comet assay with NM-102 (positive in the 1st part) was negative in three laboratories but positive in two.
Three laboratories working with Caco-2 cells had problems in reading the micronucleus slides due to presence of particle agglomerates on the microscopical slides; for the remaining three laboratories, the outcome of the micronucleus assay with NM-102 (negative in the 1st part) was negative in two laboratories and positive in one.

NM-203 (SAS)

In BEAS 2B cells, the result of the comet assay with NM-203 was negative (similarly to the results in the 1st part) in three laboratories but positive in three others. The outcome of the micronucleus assay with NM-203 in BEAS 2B cells was positive in three laboratories and negative in three laboratories (results were equivocal in the 1st part).
In the comet assay with Caco-2 cells, NM-203 (positive in the 1st part) was negative in three and positive in two laboratories. The results of the micronucleus assay with NM-203 in Caco-2 cells were split, with three positives and three negatives; conflicting results had also been obtained in the 1st part.

NM-403 (MWCNT)

The comet assay in BEAS 2B cells with NM-403 (negative in the 1st part) showed a split outcome, with three negative and three positive results. The outcome of the micronucleus assay with NM-403 in BEAS 2B cells was almost unanimous: a negative result was obtained in all laboratories except one - despite the positive result in the 1st part.
In Caco-2 cells, NM-403 yielded four negatives and one positive in the comet assay (negative in the 1st part) and three positive, one equivocal, and two negative results in the micronucleus assay (equivocal in the 1st part).

NM-110 (ZnO)

NM-110 (ZnO), tested as a candidate positive nanoparticle control, was unanimously positive in the micronucleus assay with Caco-2 cells and yielded 3 positives and 3 negatives in BEAS 2B cells. In the comet assay, 3 negatives and 2 positives were recorded in Caco-2 cells and 4 positives and 2 negatives in BEAS 2B cells.

The outcome of the round robin test is presented in the Table 3.

Partner No.	TiO_2 NM-102		SAS NM-203		MWCNT MN-403		ZnO NM-110	
	Comet	**CBMN**	**Comet**	**CBMN**	**Comet**	**CBMN**	**Comet**	**CBMN**
Caco-2 cells								
A	-	-	-	+	-	+	-	+
B	+	ANP	+	+	+	+	+	+
C	-	+	-	-	-	+	-	+
D	-	-	+	-	-	-	-	+
E		ANP		-		-		+
F	+	ANP	-	+	-	(+)	+	+
BEAS 2B cells								
G	+	+	-	+	+	+	+	+
H	-	-	+	-	-	-	+	+
I	+	(+)	+	+	+	-	+	-
J	+	-	+	-	-	-	-	+
K	+	-	-	+	-	-	-	-
L	+	-	-	-	+	-	+	-

+: Positive; (+): Equivocal; -: Negative
Grey box: not performed; ANP: Analysis could not be performed.

Table 3 – Outcome of the round robin test

Conclusions

In the first part of WP5, data on the genotoxicity of 15 MNs (4 TiO2, 4 SAS, 6 MWCNTs, and ZnO as a candidate positive nanoparticle control) were generated from the comet assay and the micronucleus assay using a number of different human cell lines of pulmonary, intestinal, and epidermal origins. In addition, micronuclei were also studied in human primary lymphocytes, and mutations in mouse lymphoma cells. While the mouse lymphoma assay was uniformly negative, the outcome of the comet assay and the micronucleus assay varied greatly among the different cell systems. It is presently unclear how much of this variation represented true differences among the cell systems and how much could be explained by experimental variations. Although dose-dependent effects could be seen in many experiments, the genotoxicity of the MNs studied was generally relatively low; in such a situation, experimental variation may determine if the result will turn out positive or negative. Variation may occur among experiments, e.g. in MN dispersions, the agglomerate size of the MN in the cell culture, MN sedimentation on the cells, and thereby cellular uptake and intracellular dose. Agglomerates of different size and shape may have differential effects on cells.

In the round robin study, relatively reproducible results were obtained for the comet assay with NM-102 in BEAS 2B cells (mostly positive) and with NM-403 in Caco-2 cells (mostly negative), and for the micronucleus assay with NM-403 (mostly negative) in BEAS 2B cells and NM-110 with Caco-2 cells (all positive). When a positive response was seen, it was low (similarly to the 1st part of WP5), which probably contributed to the situation where identical outcomes were not systematically obtained.

Although ZnO may be applicable as a nanoparticle positive control in some *in vitro* cell systems such as the micronucleus assay in Caco-2 cells, it does not appear to be universally suitable for this purpose because of the narrow dose range of its genotoxicity in some cell systems.

In summary, the present studies suggest that many MNs have some genotoxic potential detectable in human cells *in vitro* using the comet assay or the micronucleus assay. On the other hand, the mutation assay with mouse lymphoma cells appears to give only negative results.

The *in vitro* genotoxic effect of the MNs studied was mostly low, which possibly contributed to the variation observed in outcome among the cell systems. It is technically feasible to perform such genotoxicity assays

with dispersed MNs in cultured cells, but the predictive value of these *in vitro* tests in identifying MNs that are genotoxic *in vivo* that could be carcinogenic is presently unclear. More information on the mechanisms of (i) the detected *in vitro* genotoxicity and of (ii) the MNs that are genotoxic *in vivo* or carcinogenic is needed before this question can be answered.

TOXICOKINETICS AND TISSUE DISTRIBUTION OF MNS AND IDENTIFICATION OF ORGANS AT RISK FOR GENOTOXICITY TESTING

Objectives

The aim of WP7 is to identify relevant organs for genotoxicity testing based on the determination of organ exposure to nanomaterials. The distribution of the nanomaterials into the various organs is an indication for the organs at risk for nanomaterial toxicity and thus also genotoxicity, based on the interaction and activity of the nanomaterials with the cells of the organ. In WP7, the kinetic parameters and tissue distribution are determined for selected nanomaterials: titanium dioxide (TiO_2), silicon dioxide as synthetic amorphous silica (SAS), and carbon nanotubes (CNTs) after oral and intravenous administration.

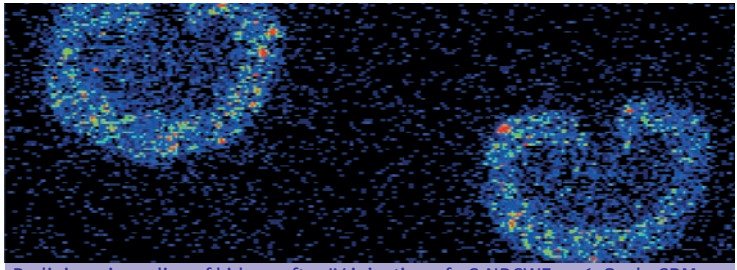
Routes investigated

The oral route of exposure was chosen as this is a common route of exposure for consumers. However, after oral exposure the absorption of MNs may vary greatly. After inhalation and dermal exposure in general the distribution and absorption of MNs were demonstrated previously to be low, if any. In addition to the oral route, the intravenous route (IV) of exposure was also investigated as this route of administration circumvents the biological barriers present and results in direct systemic availability of the nanomaterials in the blood circulation and thus in the internal organs. Organ and blood samples were collected and evaluated for detection for Ti, Si, and CNTs. For SAS and TiO_2 MNs, it was not the MNs themselves but the elements silica (Si) and titanium (Ti) which were determined using different inductively coupled plasma equipment: Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), High Resolution Inductively Coupled Plasma-Mass Spectrometry (HR-ICP-MS). For SAS, a fit-for-purpose analytical method was developed using ion-molecule chemistry to eliminate polyatomic interferences and enable interference free-detection of Si. For the CNTs the determination of C was not an option to study the tissue distribution as C is present in all tissues. Therefore, CNTs were radiolabeled with ^{14}C atoms to allow detection in the body.

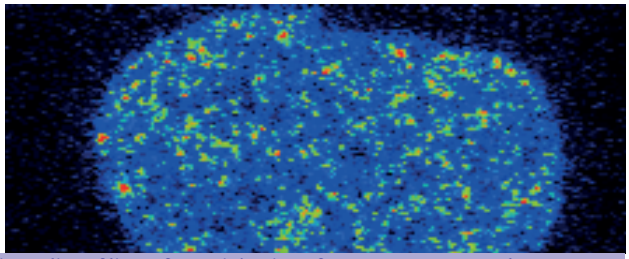
Results

For the investigated MNs the **oral administration** resulted in a low uptake from the gastro-intestinal (GI) tract even after 5 repeated oral administrations. The infrequent and incidental demonstration of some Ti in tissues beyond the GI-tract may indicate that uptake of TiO_2 is possible. In addition, it was demonstrated that the faeces of control rats already contained an amount of Ti well above the detection limit. Similar Ti levels were observed in the GI-tract of control and IV exposed rats which led to the conclusion that excretion *via* GI-tract after IV injections is not occurring. For SAS, the levels determined in liver and spleen as indicator organs for systemic uptake were similar to control levels or close to the detection limit indicating a very low absorption from the GI tract. The organ levels after repeated oral administration suggest minor differences between male and female animals and between the SAS nanomaterials (NM-200 and NM-203) investigated, although the limited absorption makes it difficult to draw any firm conclusions. Translocation of the MWCNT from the GI-tract into the systemic circulation or any of the organs investigated was not demonstrated.

For the single and repeated **IV administrations**, the results indicate that the TiO_2 MNs can remain in the body for a prolonged period of time, the exception being NM-105 (an anatase-rutile mixture). For all TiO_2, there was a rapid decrease in blood concentrations after the IV administration with most of the Ti cleared from the blood 2 hours after administration. A similar pattern was observed for both the single and repeated IV administration. The liver was the major organ for the Ti distribution followed by the spleen and the lung, while other organs investigated (brain, kidney, thymus, reproductive organs etc.) had a distribution below 0.1% of the dose administered. For some TiO_2 redistribution was noted with spleen levels increasing and liver levels decreasing. However, the liver remains the organ with the highest uptake in view of its size and total weight. It should be noted that not only the levels expressed as a percentage of the dose are important in the tissue distribution of nanomaterials, the levels



Radioimaging: slice of kidney after IV injection of ^{14}C-NRCWE-006. Scale CPM. Credit CEA.



Radioimaging: Slice of liver after IV injection of ^{14}C-NRCWE-006. Scale CPM. Credit CEA.

expressed as µg/g organ can give a direct indication of the possible exposure of the organ and might be indicative for the induction of toxicity. All five TiO_2 MNs were still present in various organs at day 90 after the IV administrations, only for one (NM-105) out of five TiO_2 was a major decline in organ levels noted. The results indicate that after the IV administration of manufactured TiO_2 there is no excretion of Ti *via* the faeces.

For the SAS, a decrease in tissue concentrations was observed between day 2/day 6 and day 90 after the repeated and single dosing indicating a clearance from the body. Major organs for the distribution of the MNs are liver, spleen and lung and to a lesser extent the kidney. For SAS, following single dose IV administration, measurable concentrations slightly above the limit of quantification of Si were detected in the liver of male and female rats up until 90 days after administration. After repeated IV administration, a considerable concentration of Si is present in liver and spleen of males at day 6, with marked particle- and gender-related differences, and detectable concentrations found in other organs as well. After a single IV SAS administration, the highest level of NM-203 was observed in the spleen of male rats, while for NM-200 in male and female rats the highest concentration was noted in the liver. In female rats the concentration of NM-203 was similar for liver and spleen. After repeated IV SAS administration NM-203 showed the highest concentrations in the spleen of male rats, while for NM-200 the highest concentration was in the liver. In female rats, similar concentrations were observed in liver and spleen both at day 6 and day 90 for NM-203, while for NM-200 the highest concentration was present in the liver both at day 6 and day 90. The meaning of such differences is not clear. However, although there is a clear decrease in Si concentration in liver and spleen at day 90 after intravenous administration, Si concentrations were still distinctly higher than in controls suggesting that a longer time period than 90 days is required for complete elimination of administered SAS from the body.

Each type of the four MWCNTs investigated was found to display particular bioaccumulation and biopersistence properties in the various organs evaluated. NM-400 and NRCWE-006 showed much higher bioaccumulation than NM-401 and NM-402. In liver and spleen, at day 90, a marked reduction in the MWCNT level expressed as percentage of injected dose, was only observed for NM-400. For NM-401, NM-402 and NRCWE-006 no significant decrease was observed between day 6 and day 90. In lung, a significant reduction in the MWCNT level expressed as percentage of injected dose was only observed for NM-401. Although a decrease was observed between day 6 and day 90 for NM-400, all MWCNTs investigated including NM-400, were still observed in the various organs (liver, spleen, lung) at day 90 after the administration. For NM-401, NM-402 and NRCWE-006, the data suggest a significant biopersistence of these MWCNTs in most organs beyond 3 months after administration.

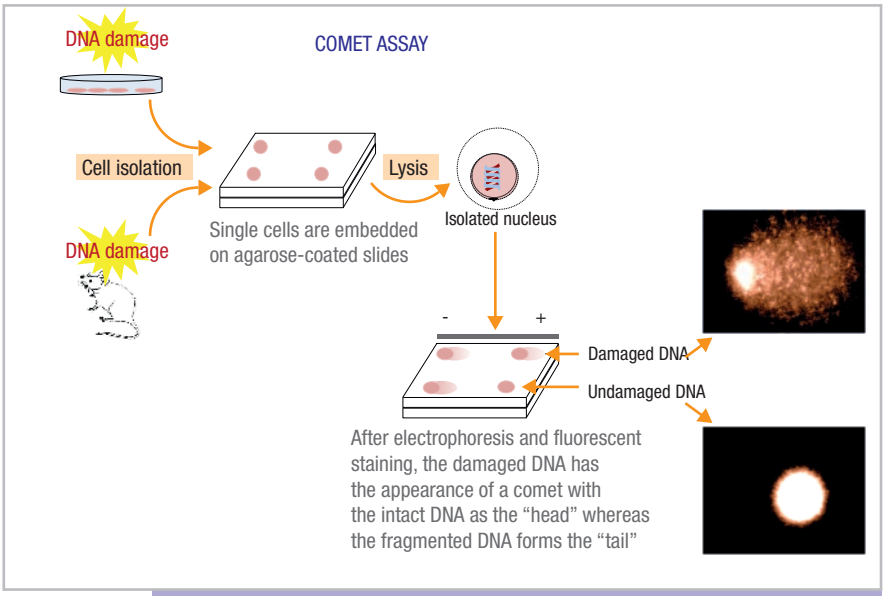
Although the detection of the radiolabel is indicative for the presence of the CNT in organs it could also be that a detached label not associated with the CNTs was measured. In additional experiments it was demonstrated unambiguously that the radioactive signal indeed corresponds to the presence of CNTs carbon nanotubes, with a direct visualisation of the CNT walls, the walls inside, and finally measurement of the CNT diameter.

Conclusion

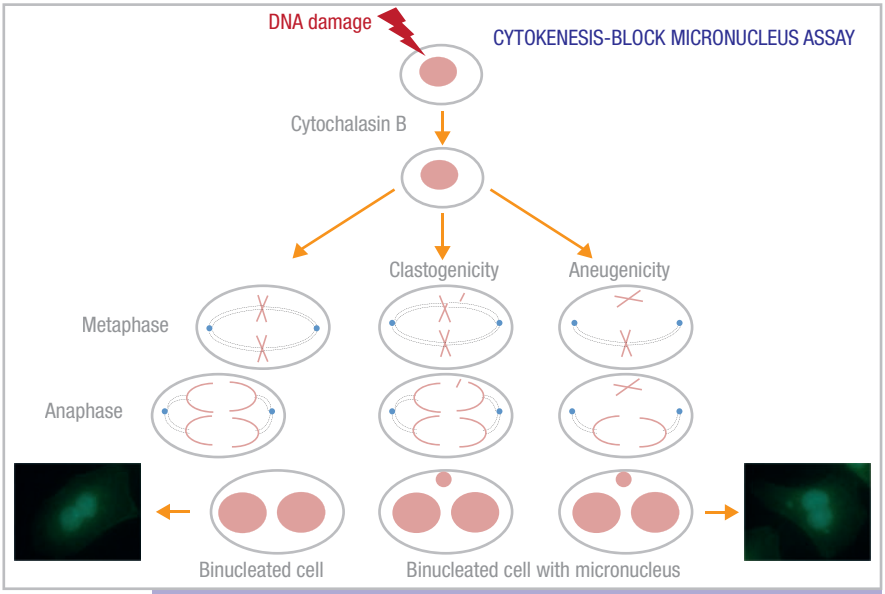
For all TiO_2 and SAS nanomaterials, oral administration resulted in a rather low uptake *via* the GI-tract after repeated oral administration, whereas for MWCNT no uptake from the GI-tract was demonstrated.

After IV administration, most MNs showed a rapid clearance from the blood indicating a quick distribution to, and uptake by, the various organs. For the single and repeated IV administrations, the results indicate that especially some TiO_2 and MWCNT are still present in the organs at day 90 after the last administration. For SAS in general, a decrease in Si level was noted between day2/day 6 and day 90, although at day 90 Si could still be detected.

Major organs for the biodistribution of the investigated MNs are liver, spleen and lung and to a limited extent the kidney. Although the IV administration can be considered an artificial route of exposure, the results obtained clearly demonstrate that some MNs can persist in organs for a prolonged period of time until at least 90 days, the last time point investigated in these studies.



Comet assay



Micronucleus assay

IN VIVO GENOTOXICITY TESTING

Toxicological studies require experimental models, *in vitro* (organs or cell cultures) or *in vivo* (animals, mostly rodents). *In vitro* methods are increasingly developed as an alternative approach to animal experimentation due to simplicity and rapidity, along with cost effectiveness and animal welfare considerations. *In vitro* models are also useful to elucidate the mechanisms of toxicity induced by xenobiotics. However, *in vivo* studies in rodents will reflect the toxicokinetics (uptake and behaviour of a xenobiotic in the whole body), taking into account the complexity of a whole organism. For risk assessment, *in vivo* results still remain unavoidable.

Objectives

The aim of WP6 was to complete the results obtained on *in vitro* models by *in vivo* genotoxicity testing, using comet and micronucleus assays in rodent models. Correlation between *in vivo* and *in vitro* results should be assessed taking into account the toxicokinetic results.

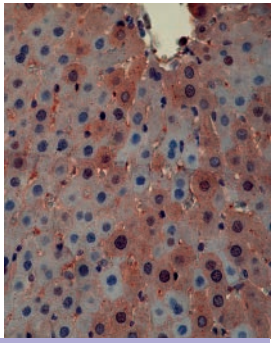
Methods

In vivo studies were conducted on male rats exposed to three doses [the highest concentration being the non toxic dose used in WP7 plus 2 lowest doses (dilution/2)] of nanoparticles suspensions (up to 5 animals per dose). Two routes were investigated: instillation and gavage. In order to detect genotoxic effects, an administration schedule of 3 consecutive days with tissue sample collection 3 hours after the last administration was chosen. The doses were selected according to the toxicokinetics data from WP7, where no death or obvious adverse effect was induced: up to 20 mg/kg/day SAS, 12.8 mg/kg/day TiO_2 and 51.2 mg/kg/day CNTs for gavage and up to 12 mg/kg/day SAS, 4.6 mg/kg/day TiO_2 and 0.48 mg/kg/day CNTs for instillation. Depending on the route of exposure, up to 6 organs/tissues were collected for comet genotoxicity testing: liver, kidney, blood, bone marrow, intestine and colon for the oral route while intestine and colon were replaced by lung and bronchoalveolar lavage (BAL) fluid for the instillation route. From the same animals, bone marrow (according to the OECD guideline n°474[2]) and colon samples (embedded in paraffin) were also studied by the micronucleus assay. A piece of organs was also kept for further histology analysis in case

2. OECD Test Guideline No. 474: Mammalian Erythrocyte Micronucleus Test

of positive results in the comet assay. To measure the inflammation following instillation of nanoparticles, BAL fluid cytology was also performed. Oxidative DNA damage was also investigated using a modified comet assay with FpG enzyme recognising some specific oxidative lesions. Methylmethane sulfonate (MMS) and N-ethyl-N-nitrosourea were used as chemical positive controls.

Various methods for cell isolation (enzymatic, mechanical) were used according to the protocol set up in each laboratory. At least one hundred cells per organ per animal were scored for comet assay (parameters: % tail DNA, tail moment). For micronucleus assays, at least 2000 immature erythrocytes or 1000 colon crypt cells were scored per animal. Statistical analysis was performed with the non-parametric Kruskall-Wallis test for the comet assay and the chi-square test for micronucleus assays.



Immunohistochemical localisation of a metallothionein (in brown) in the liver of Wistar rats treated i.v. with 11 mg/kg b.w NM-105 for 5 consecutive days. (Magnification x200). Credit IMB-BAS.

A gene mutation assay on LacZ mice was also performed with NM-102 (TiO_2) according to the transgenic rodent mutation assay OECD guideline n° 488. It was selected according to the *in vitro* genotoxicity and toxicokinetics results. Animals were treated intravenously with NM-102 (10 and 15 mg/kg b.w.) for 2 consecutive days. Following 28 days, the DNA of the target organs (liver and spleen) was extracted and the mutant frequencies were determined. In this integrative mouse study, comet assay on liver and spleen as well as bone marrow micronucleus assay were also performed. Furthermore, to verify that the exposure of the mice in the organs was effective, samples from liver tissue were also collected for Transmission Electron Microscopy (TEM) and histopathology analyses.

Results

TiO_2

After instillation, only one (NM-105) out of the four TiO_2 nanomaterials induced DNA damage in BAL cells. Two other TiO_2 (NM-102 and -103) gave equivocal dose responses in liver. None of the TiO_2 nanomaterials studied showed genotoxic effects in lung, spleen, and kidney.

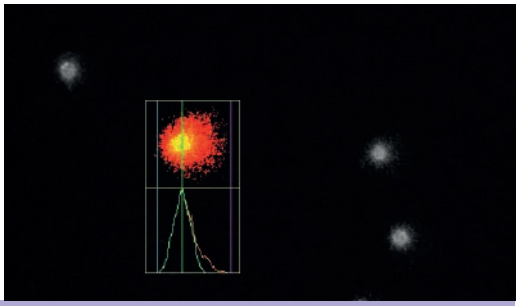
Following gavage, some genotoxic effects were observed with the comet assay with TiO_2 in spleen, intestine (NM-103), colon (NM-102 and -105). However, all TiO_2 nanomaterials studied showed no genotoxic effects in liver samples.
Additional studies were included from both after single and repeated (5 times) intravenous administrations for NM-103 and NM-104 in order to increase the MNs potency to reach systemic organs, no increase in micronuclei could be detected in bone marrow. Similar negative results for bone marrow micronucleus assay after repeated IV (2 times) exposure to NM-102 were obtained from the lacZ mice assay. Moreover, no genotoxic effects (comet assay) could be disclosed for NM-102 in liver and spleen and no mutagenic effect was observed in liver and spleen from lacZ transgenic mice.

SAS

No obvious DNA damage was detected with the comet assay for the four SAS tested whether after oral or instillation exposure. Moreover, no specific oxidative DNA damage was detected using the modified FpG comet assay. The genotoxicity of one SAS (NM-203) was also investigated after intravenous exposure in order to increase the bioavailability of MNs to systemic organs but no DNA damage was induced irrespective of the organ or tissue, even when using FpG.
None of the four SAS induced micronucleus formation in bone marrow after gavage. For instillation, no induction of micronuclei in bone marrow was detected irrespective of the SAS. For intravenous administration (NM-203 only), results were also negative, even though a slight increase in micronucleus formation was observed at the highest dose tested (20 mg/kg), but which induced also some animal death (three out of six). Oral administration of two (NM-202 and -203) out of the four SAS induced an increase of micronuclei in colon samples but only at the lowest dose (5 mg/kg).

CNTs

After gavage, some equivocal dose-responses from the comet assay were obtained for NM-401 in liver and kidney while the results were negative for the other organs collected. Results from FpG modified assay did not show any specific oxidative damage irrespective of the CNT.
After instillation, an obvious induction of DNA damage was only noticed in kidney for NM-400 and in BAL for NM-401. An equivocal dose-response was obtained in spleen for NM-401 and in BAL, lung and kidney for NM-403. Results from FpG modified assay indicated some oxidative DNA



Big comet in kidney (with FpG). Credit ANSES.

damage principally in kidney samples for NM-401 and NM-402.
No induction of micronuclei was reported in the bone marrow after instillation exposure for the four CNTs.

Conclusion

With the comet assay, the responses were largely negative for most of the MNs tested and the organs considered. In most cases, when positive results were obtained, no dose response relationship could be established which makes it difficult to conclude on the *in vivo* genotoxicity of the MNs tested.
The WP6 results showed that no mutation damage was observed in bone marrow after gavage with either of four SAS, which may be explained by the low bioavailability of SAS after gavage (as observed in the toxicokinetics studies) or by SAS dissolution. None of the tested TiO_2, SAS and CNT nanomaterials induced micronuclei formation in bone marrow after instillation and gavage while two SAS (NM-202 and -203) induced an increase of micronuclei in colon samples but only at the lowest dose.

Recommendations

It should be noted that, in order to reduce the number of animals within this WP, the two genotoxic assays (Comet and micronucleus assay) were performed together. As MNs were administered on 3 consecutive days and more than 5 tissues from the same animal were collected, a good organization was required. Moreover, performing non-OECD genotoxicity assays can be challenging and would require agreeing on criteria for data acceptability. In order to reduce the large intra- and inter-laboratory variabilities observed, it is recommended that only experienced laboratories would conduct this specific *in vivo* genotoxicity testing. Finally, the internalization of MNs in some key organs must be confirmed to correlate those data with the genotoxic ones.

CONSIDERATIONS TO ACHIEVE A ROBUST METHOD FOR GENOTOXICITY TESTING OF MNs

In the risk assessment process, the first step of hazard identification is the process of determining whether exposure to a stressor can cause an increase in the incidence of specific adverse health effects, it is therefore necessary to study the effects associated with different routes of exposure. In NANOGENOTOX, the effects on cell lines corresponding to the different routes were studied.

The JA demonstrated that the tests used for chemical genotoxicity testing (i.e., OECD TG 487 Guideline for the testing of chemicals *in vitro* mammalian cell micronucleus test) are applicable for nanomaterials but may need some adaptation in order to provide predictive results *in vivo*.

The JA demonstrated the need to conduct a complete and reliable physico-chemical characterisation both of the bulk and the dispersed material. The JA demonstrated that it is not possible to classify as "mono-substance" the families of MN studied (TiO_2, SAS and MWCNTs) as non negligible differences were observed in the physico chemical characterisation, in the results of the *in vitro* and *in vivo* genotoxicity as well as in the toxicokinetic behaviour.

It was agreed that though several physico-chemical characterisation methods are available, some perform better than others depending on the parameters studied. For example to determine the primary MN size-distribution, TEM was strongly recommended, however SAXS may be applied for average size while DLS is very useful for evaluation of dispersion quality and stability in MN suspensions.

The MNs investigated in NANOGENOTOX did not so far show strong genotoxicity *in vivo* or *in vitro*; neither in exposed target cells following gavage (colon), nor after instillation (lung), nor *in vitro* on 3D reconstructed human skin models. However, in several cases, even at the lowest tested doses, some genotoxic effects were detectable *in vitro* and *in vivo*.

For hazard identification of substance-related genotoxicity, the OECD test guideline TG 487 can be used but with target cells corresponding to the route of exposure. However, particle uptake into the cells of the chosen test system should be demonstrated, otherwise negative results might occur due to lack of exposure, hence not describing the potential hazard. The *in vivo* mammalian erythrocyte micronucleus OECD test guideline (TG 474) can also be used, however, similarly, it must be demonstrated that the test item reaches the target cells *in vivo*. NANOGENOTOX also highlighted the necessity of toxicokinetic studies in this context.

The results of this JA also suggest that other *in vivo* tests might be ap-

plicable for genotoxicity investigation of MNs, for example the *in vivo* micronucleus assay on lung, or intestine cells or colon as some genotoxic effects were observed *in vivo* on those organs.

RECOMMENDATIONS AND PERSPECTIVES

- In general and according to the behaviour of the MNs and their specificities, any genotoxic test guideline should be amended to include some toxicokinetic testing as there is a critical need to always investigate whether the tested nanomaterials reach the target cells and not just rely on genotoxicity methods commonly used.
- The variability in the results observed in the outcome of the tests used in *in vitro* and *in vivo* studies highlighted the need to include historical data or criteria of acceptability and reproducibility of testing, especially for non-OECD tests and with cell models which are not commonly used for genotoxicity investigations. However it was highlighted that this variability of the results is also seen for chemicals, particularly in genotoxicity testing.
- Even if these tests are commonly used for genotoxicity it was demonstrated that a sufficient level of skills is required to perform the testing (including for instance, preparation and scoring of the slides as well as the results interpretation).
- In addition, any genotoxicity assessment *in vitro* should specify the dispersion protocol used to prepare the MNs and the characterisation of the resulting dispersion, and provide information on the availability of the MNs to reach the cells/tissues and their uptake. The most sensitive and relevant cell type according to the relevant exposure route should be used, and appropriate positive and negative controls should be included.

The participation of a large number of scientific teams from various EU Member States enabled the development of a common methodology and should contribute to its uptake and implementation. In the institutes participating in the JA, researchers, post-doctoral fellows and PhD students were trained in the experimental procedures developed for the JA, and will in the future contribute to the dissemination of SOPs and protocols. Several ministries of different EU Member States participated

in the JA as collaborating partners and followed the progression of the JA's work as well as its outcomes thus contributing to the sustainability of the action.

The JA has contributed to the creation of a network of laboratories within the partner institutes that will hopefully continue to work together. This should allow results from genotoxicity testing to be shared and accepted by different Member States thereby avoiding unnecessary duplication. The JA accelerated the exchange of best practices in *in vitro* and *in vivo* genotoxicity as well as in the physico-chemical characterisation of manufactured nanomaterials. The developed method (data and results obtained will be publically available) may be used by the Member States and EU human health risk assessment and regulatory bodies, industries, consumer or worker protection associations and others, thereby improving public health in the EU.

LIST OF DELIVERABLES

Deliverable no.	Deliverable title	WP	Dissemination level
1	Evaluation reports	3	CO
2	SOPs for characterisation of the selected MN types	4	PU
3	Final protocol for producing suitable MN exposure media	4	PU
4	MN data sets with requested physico-chemical properties	4	PU
5	*In vitro* genotoxicity testing strategy for nanomaterials including database	5	PU
6	Characterisation of MNs for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis	6	PU
7	Identification of target organs and biodistribution including ADME parameters	7	PU
8	Report for stakeholders on the JA results and policy recommendations	2	PU
9	Interim and final technical and financial reports	1	CO

CO: Confidential

PU: Public

GLOSSARY

Abbreviations

ADME: Absorption, Distribution, Metabolism, Elimination

ANSES: Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail – French Agency for Food, Environmental and Occupational Health & Safety

BAL: Bronchoalveolar lavage

BfR: Bundesinstitut fur Risikobewertung - Federal Institute for Risk Assessment

BSA: Bovine Serum Albumin

CEA: Commissariat à l'énergie atomique et aux énergies alternatives

CEN: European Committee for Standardization

CNT: Carbon Nanotube

CODA-CERVA: Centrum voor Onderzoek in Diergeneeskunde en Agrochemie – Centre d'étude et de recherche vétérinaire et agrochimique

DG SANCO: European Commission's Directorate General for Health and Consumers

DLS: Dynamic Light Scattering

DNA: DeoxyriboNucleic Acid

EC: European Commission

EAHC: Executive Agency for Health and Consumers

EU: European Union

FIOH: Finnish Institute of Occupational Health

FpG: formamido–pyrimidine–DNA–glycosylase

GI: Gastro Intestinal

HPLC-ICP-MS: High-performance liquid chromatography

HR-ICP-MS: High Resolution Inductively Coupled Plasma Mass Spectroscopy

ICP-MS: Inductively Coupled Plasma Mass Spectroscopy

ICP-OES: Inductively Coupled Plasma Optical Emission Spectroscopy

IMB-BAS: Institute of Molecular Biology "Roumen Tsanev" – Bulgarian Academy of Sciences

IMC-BAS: Institute of Mineralogy and Crystallography – Bulgarian Academy of Sciences

INRS: Institut national de recherche et de sécurité pour la prévention des accidents du travail et des maladies professionnelles

INSA: Instituto Nacional de Saúde Dr. Ricardo Jorge

IPH: Scientific Institute of Public Health

IPL: Institut Pasteur de Lille

ISO: International Standardization Organisation

ISS: Istituto Superiore di Sanità - Italian National Health Institute

IV: Intra Venous

JA: Joint Action

JRC: Joint Research Centre

LNE: Laboratoire national de métrologie et d'essais

MN: Manufactured Nanomaterial

MWCNT: Multi-Walled Carbon Nanotubes

NGO: Non–Governmental Organisation

NIOM: Nofer Institute of Occupational Medicine

nm: nanometer

NRCWE: National Research Centre for the Working Environment

OECD: Organisation for Economic Co-operation and Development

WPMN: Working Party on Manufactured Nanomaterials

REACH: Registration, Evaluation, Authorization of Chemicals

RICC: relative increase in cell counts

RIVM: Rijksinstituut voor Volksgezondheid en Milieu – National Institute for Public Health and Environment

RPD: relative population doubling

RSA: Rat Serum Albumin

SAS: Synthetic Amorphous Silica

SAXS: Small Angle X-ray Scattering

SWCNT: Single-Walled Carbon Nanotubes

SiO_2: Silicon Dioxide

SOP: Standard Operating Procedure

TEM: Transmission Electron Microscopy

TiO_2: Titanium Dioxide

UAB: Universitat Autonoma de Barcelona

UCD: University College Dublin

USAXS: Ultra Small Angle X-ray Scattering

WP: Work Package

Definitions

ADME parameters: set of parameters used in pharmacokinetics, a branch of pharmacology dedicated to the determination of the fate of substances administered to a living organism. Pharmacokinetics is divided into several areas which includes the extent and rate of Absorption, Distribution, Metabolism and Excretion. This is commonly referred to as the ADME scheme. Pharmacokinetics describe how the body affects a specific drug after administration. The site of administration and the concentration in which the drug is administered could affect its pharmacokinetic properties.

Agglomerate: collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Note 1 The forces holding an agglomerate together are weak forces, for example van der Waals forces, or simple physical entanglement.

Note 2: Agglomerates are also termed secondary particles and the original source particles are termed primary particles. (ISO TS 27687:2008).

Aggregate particle: comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components.

Note 1: The forces holding an aggregate together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement.

Note 2: Aggregates are also termed secondary particles and the original source particles are termed primary particles. (ISO TS 27687:2008).

Aneugenic effect: action affecting cellular division and inducing an abnormal separation of the chromosomes, resulting in the formation of cells with an abnormal number of chromosomes (aneuploidy).

Carbon nanotubes: cylindrical tube-like structures elaborated from graphite sheets. Some exhibit remarkable properties including: mechanical properties (strength, rigidity, flexibility, etc.), physio chemical properties (good thermal or electrical conductivity, etc.). Carbon nanotubes are typically a few nanometres in diameter and several micrometres to centimetres long. Different types of CNT are produced according to the wrapping of the graphite sheet(s). A carbon nanotube that has only one layer of graphite is called a single walled carbon nanotube (SWCNT) and nanotubes that consist of multiple layers (concentric tubes) of graphite are called multiwalled carbon nanotubes (MWCNT).

Carcinogenic: capable to induce, promote or aggravate cancers.

Clastogenic effect: effect that can cause breaks in the chromosomes.

Comet assay: also called Single Cell Gel Electrophoresis (SCGE) is a rapid, simple, visual, and sensitive technique for measuring and analysing DNA breakage in individual cells.

Manufactured nanomaterial (MN): nanomaterial intentionally produced to have specific properties or composition (ISO/CD TS 80004-1, under publication).

Mutagenic: that can cause modifications in the nucleic acid sequence (DNA).Mutations can be natural, but can also be caused by mutagenic agents and can be responsible for cancers.

Nanomaterial: material with any external dimension in the nanoscale or having internal or surface structure in the nanoscale.

Note: Generic term covering both nano-object and nanostructured material. (ISO/CD TS 80004-1, under publication).

Nanometer: one billionth of a meter (1/1,000,000,000 or 1. 10-9 m). Abbreviation for nanometer: nm.

Nanoparticle: nano-object with all three external dimensions in the nanoscale.

Note: If the lengths of the longest to the shortest axes of the nano-object differ significantly (typically by more than three times), the terms nanofibre or nanoplate are intended to be used instead of the term nanoparticle. (ISO TS 27687:2008).

Nanoscale: size range from approximately 1 nm to 100 nm.

Note 1: Properties that are not extrapolations from a larger size will typically, but not exclusively, be exhibited in this size range. For such properties the size limits are considered approximate.

Note 2: The lower limit in this definition (approximately 1 nm) is introduced to avoid single and small groups of atoms from being designated as nano-objects or elements of nanostructures, which might be implied by the absence of a lower limit. (ISO TS 27687:2008)

REACh: Registration, Evaluation, Authorisation of Chemicals. REACh is the new EC regulation no. 1907/2006 that has been in force since June 1, 2007.

Genotoxicity: describes a harmful action on a cell's genetic material affecting its integrity. Genotoxic substances are known to be potentially mutagenic or carcinogenic, specifically those capable of causing genetic mutations and of contributing to the development of tumors. This includes certain chemical compounds but also certain types of radiation.

Round robin test: also called ring test, is a test (measurement, analysis, or experiment) performed independently several times. For example in the NANOGENOTOX JA, it involves multiple independent scientists

performing the same test with the use of the same protocols in different laboratories. The aim of the round robin tests is to evaluate the robustness of the developed methodology by using different equipment and apparatus

SOPs: Standard Operating Procedures: A set of instructions covering those features of operations which lend themselves to a definite or standardized procedure without loss of effectiveness.

Silicon dioxide: also known as silica, is an oxide of silicon (chemical formula SiO_2). Crystalline silica is naturally widely abundant in sand and soils, rocks (sandstone, granite), minerals (quartz). Amorphous silica is far less abundant in nature (this form is called diatomite) but is commonly manufactured and called SAS by industry for Synthetic Amorphous Silica. In the NANOGENOTOX Joint Action, SAS or silica only refers to the nanometric SAS (micronised agglomerates of SAS nanoparticles). Nanometric SAS have been mass produced since 1950 and widely used for a large range of industrial applications (flow agents, anti-cacking agents and flavor carriers in food, polishing agents in toothpastes, flattening agents and thickeners in paints, etc.).

Titanium dioxide: is the naturally occurring oxide of titanium (chemical formula TiO_2). Three natural crystalline structures are identified: rutile, anatase and brookite. Among them only rutile and anatase are commonly used due to their stability and their natural abundance. Anatase is mainly used at the nano-scale for its remarkable photocatalytic properties for anti-microbial and self cleaning material applications while rutile nanomaterials are mainly used for their optical properties (e.g.: UV-block for sunscreen and paint).

Stakeholders: in the JA, this term describes an individual or a group that is concerned or stands to be affected – directly or indirectly – by NANOGENOTOX's work.

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BfR		
CEA		
CODA-CERVA		
FIOH		
IMB-BAS		
IMC-BAS		
INRS		

Unit/laboratory	WP implication
Risk Assessment Department	**WP6**, WP7
European and International Affairs Department	**WP1**, WP2
Department of Information, Communication and Dialogue with Society	**WP2**
Fougères Laboratory - Toxicology of Contaminants Unit	WP5, **WP6**
Fougères Laboratory - Pharmacokinetic-pharmacodynamic Unit	WP7
Maisons-Alfort Laboratory for Food Safety - Environmental Inorganic Contaminants and Mineral Unit	WP7
Molecular toxicology unit of the Department of Safety of Consumer Products	**WP3**, WP5
Materials Sciences Division - Saclay Institute of Matter and Radiation (IRAMIS)	WP4
Life Sciences Division - Institute of biology and technology of Saclay (iBiTec-S)	WP7
Electron Microscopy unit	WP4
New Technologies and Risks laboratory	**WP5**, WP6
Medical and Biological Research Laboratory	WP5, WP6, WP7
Institute of Mineralogy and Crystallography	WP4
Aerosol Metrology Laboratory and the Inorganic Analysis and Aerosol Characterization Laboratory	WP4
Carcinogenesis and Developmental Toxicology Laboratory	WP5, WP6
Technical expertise and consulting division	WP2

Partner's acronym	Logo
INSA	
IPH	
IPL	
ISS	
NIOM	
NRCWE	
RIVM	
UAB	

	Unit/laboratory	WP implication
	Group of Genetic Toxicology, R&D Unit	WP5, WP6
	Laboratory of toxicology	WP5
	Genetic Toxicology Laboratory	WP5, WP6
	Food and Veterinary Toxicology Unit	WP7
	Laboratory of Molecular Toxicology	WP5, WP6
	National Research Centre for the Working Environment	**WP4**, WP5, WP6, WP7
	Laboratory for Health Protection Research	**WP7**, WP5, WP6
	Group of Mutagenesis	WP5

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