Towards a method for detecting the potential genotoxicity of nanomaterials

Deliverable 5: In vitro testing strategy for nanomaterials including database

Final report

In vitro testing strategy for nanomaterials including database
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WP 5: *In vitro* methods for genotoxicity

Deliverable 5: *In vitro* testing strategy for nanomaterials including database

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**Workflow**

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Methodology

1st part: generation of in vitro genotoxicity data

WP5 investigated the in vitro genotoxicity of MNs. In the 1st part of WP5 (genotoxicity data generation), three complementary genotoxicity tests were performed in various cell systems:

- The alkaline comet assay (early DNA damage)
- The micronucleus assay (structural and numerical chromosome alterations)
- The mouse lymphoma assay (mutations).

Various human cell lines of different origin were used for the comet and the micronucleus assays: pulmonary (bronchial epithelial BEAS 2B and 16 HBE; adenocarcinomic human alveolar basal epithelial A549), intestinal (epithelial colorectal adenocarcinoma Caco-2, primarily undifferentiated cells used) and epidermal (NHEK). In addition, the comet assay was also performed in a 3-dimensional human reconstructed full thickness skin model. The micronucleus assay was also performed in human primary lymphocytes, a cell system widely used in genotoxicity testing of soluble chemicals.

The alkaline comet assay, a simple and sensitive method for the detection of DNA strand breaks (single- and double-strand breaks), alkali-labile sites, and excision repair sites, was used in all the human cell systems applied in the 1st part of WP5. In some laboratories, a modified comet assay based on the use of FpG (formamidopyrimidine-DNA-glycosylase), capable of improved detection of oxidative DNA damage, was voluntarily used as a supplementary assay.

Figure 1. **Left:** 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 (the green, purple and white colour were created by the computer program; credit IMB-BAS). **Right:** 3-D full thickness skin model (EpiDermFT™) (credit BfR).

The micronucleus assay - able to detect agents that modify chromosome structure or segregation, leading to the formation of an additional nucleus (micronucleus) during cellular division - was applied to all of the human cell systems used, except the reconstructed full thickness 3-D skin model, where micronucleus detection was finally not deemed practicable with the present techniques. The OECD guideline on the in vitro micronucleus test was, in most parts, used as the basis for the micronucleus
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assays performed. The cytokinesis block micronucleus assay, using cytochalasin B (Cyt-B) to prevent cytokinesis, was performed in all cell lines, except 16 HBE cells.

Figure 2. Fluorescence micrographs of human Caco-2 cells stained with (left) DAPI (4',6-diamidino-2-phenylindole) and (right) acridine orange. DNA is stained blue by DAPI and green by acridine orange. Micronuclei are scored in cells that have divided once after the MN treatment; in the cytokinesis block micronucleus assay, these cells are binucleate because their cytokinesis has been inhibited by cytochalasin B. Both figures show a binucleate cell containing a micronucleus (credit ANSES).

In addition, all MN were also tested in the mouse lymphoma assay, performed in mouse lymphoma L5178Y TK+/- cells. This technique is able to detect a wide spectrum of mutations, including gene mutations.

The In vitro comet and micronucleus assays, complemented with the mouse lymphoma assay, were applied to all of the three categories of MNs assessed: TiO₂, synthetic amorphous silica (SAS), and multiwall carbon nanotubes (MWCNTs). However, in the epidermal systems (including the reconstructed full-thickness skin model) only TiO₂ was tested. Nanosized ZnO was included as a nanospecific positive control substance. The dispersion protocol provided by WP4 was used in all studies.

All experiments also comprised assay-specific (chemical) positive controls: mitomycin C or methyl methane sulphonate (MMS) in the micronucleus assay, ethyl methane sulphonate or MMS in the comet assay, and methyl methane sulphonate in the mouse lymphoma assay.

The protocols were harmonized, following general principles described below. One experiment cultures was performed per MN per genotoxicity endpoint. Each treatment was conducted as duplicate cultures. Some of the Partners performed two or more independent experiments. No metabolic activation systems were utilized. 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 (for Caco-2 24 h) after the start of the treatment. The doses of the MNs tested were chosen on the basis of cytotoxicity measurements using cell count relative to control, relative increase in cell counts (RICC), or relative population doubling (RPD), as well as cell viability tests based on bioreduction of XTT (sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium) and neutral red uptake. The highest dose was either at the cytotoxicity limit of 55% ± 5% or otherwise justified. For each MN, 4-6 doses were included in the genotoxicity assays to obtain a minimum of 3 analyzable doses. In the case of MNs with low cytotoxicity, the maximum dose was derived from the WP4 dispersion protocol (256 µg/ml; in some
cases double this dose) 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/cm².

Some supplementary measurements were also performed. Release of cytokine IL8, a mediator of inflammation, into Caco-2 cell culture medium was measured after a 24-h treatment by ELISA (enzyme-linked immunosorbent assay).

2nd part: round robin test

In the context of developing 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 alkaline comet assay and the micronucleus assay were chosen for the round robin study. Cyt-B was used in all of the in vitro micronucleus assays of the round robin study.

The partners were divided into two groups, one group of six laboratories using human bronchial epithelial BEAS 2B cells, and the other six laboratories using human epithelial colorectal adenocarcinoma Caco-2 cells. The round robin study comprised in vitro genotoxicity testing of one type of each family of studied MNs (doses were selected on the basis of results from the 1st part of WP5):

- TiO₂ NM-102 (doses: 0, 64, 128 and 256 µg/ml),
- SAS NM-203 (doses: 0, 8, 32 and 64 µg/ml for BEAS 2B; 0, 64, 128 and 256 µg/ml for Caco-2), and
- MWCNT NM-403 (doses: 0, 64, 128 and 256 µg/ml).

ZnO NM-110 (doses between 1.5 and 8.55 µg/ml used) was included in all series, to further assess its possible use as a nanoparticle positive control. In addition, chemical positive controls were applied in all experiments.

Positive result was defined as follows: a statistically significant increase with ≥2 doses or a statistically significant increase at the high dose and a dose-dependent increase. A statistically significant result at a single (not the high dose) was considered a weak positive result. For the comet assay, Kruskal-Wallis

Figure 3. Human bronchial epithelial BEAS 2B cells used in the round robin study. (A) A phase-contrast micrograph of BEAS 2B cells after a 48-h growth in 6-well plates. (B) A fluorescence micrograph of BEAS 2B cells stained with acridine orange for micronucleus scoring. (C) A (black and white) fluorescence micrograph of the comet assay with BEAS 2B cells treated with the positive control (EMS, 0.75 mM) (Credit INSA).
test or analysis of variance were used for examining statistical significance. For the micronucleus assay, the Chi-square test or Fisher’s exact test were applied. Dose-responses were assessed by linear regression.

Results

1st part: generation of in vitro genotoxicity data

TiO₂

Comet assay

The types of TiO₂ tested in all cell systems were NM-102, NM-103, NM-104, and NM-105. In addition, NM-100 and NM-101 (with larger and smaller particle size, respectively, than the other TiO₂ MNs) were assessed in supplementary studies performed in some cell systems. The results of the comet assays with the different types of TiO₂ tested in the various cell systems are shown in Figs 4-10.

In Caco-2 cells, the comet assay after the 24-h treatment gave positive results for all forms of TiO₂ except NM-104 (Fig. 8).

Results of the comet assay were positive, with the 3-h or 24-h treatment, for NM-102 in all cell lines and for NM-105 in all cell lines except BEAS 2B (Figs 4-9). With NM-102, both treatment times yielded a positive outcome in BEAS 2B cells, while only the 3-h treatment was positive in A549 and 16 HBE cells and the 24-h treatment in Caco-2 cells. NM-105 was positive in the 24-h treatment in Caco-2 and 16 HBE cells and in the 3-h treatment in A549 cells. In 16 HBE cells, where also NM-100 and NM-101 were tested for DNA damage, NM-100 showed a positive result (Fig. 5).

In the 3D human reconstructed full thickness skin model, all TiO₂ MNs (NM-102, NM-103, NM-104, and NM-105) investigated in the comet assay were negative (Fig. 10). In contrast, the chemical positive control MMS consistently generated a significant increase in DNA-damage (data not shown). The highest TiO₂ 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 TiO₂ through the stratum corneum of the reconstructed human full thickness skin models even after 72 h of exposure. This pointed to an undisturbed skin barrier in these 3D models which may explain the lack of positive results as compared with results obtained in NHEK cells.

As TiO₂ 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 MNs reaching dividing cells of an intact 3D skin barrier was considered close to zero. Furthermore, the full thickness skin models appear to show only a low cell division rate at the end-differentiated stage. Thus, it is postulated that MNs 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.
Figure 4. Comet assay in BEAS 2B cells with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 3-h and 24-h treatment. Symbols show mean of the medians of duplicate cultures, error bars SE (data from NIOM).
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Figure 6. Comet assay in 16 HBE cells with TiO$_2$ NM-103, NM-104, and NM-105 after 3-h and 24-h treatment. Symbols show mean of the medians of duplicate cultures, error bars SE (Data from BfR).
Figure 7. Comet assay in A549 cells with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 3-h and 24-h treatment. Symbols show mean of the medians of duplicate cultures, error bars SE (data from NIOM).
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Figure 8. Comet assay in Caco-2 cells with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 3-h and 24-h treatment. Symbols show mean of the medians of duplicate cultures, error bars SE (data from NIOM).
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Figure 9. Comet assay in NHEK cells with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 3-h and 24-h treatment. Symbols show means of 2-4 parallel cultures, error bars SE (data from IMB-BAS).
Figure 10. Comet assay in reconstructed full thickness skin model with TiO₂ NM-102, NM-103, NM-104, and NM-105 after 72-h treatment. Symbols show means of medians from 7-10 independently exposed tissues, error bars SE (Data from BfR).
Micronucleus assay

The results of the micronucleus assay with TiO$_2$ are shown in Figs 11-16.

The micronucleus assay was positive for each TiO$_2$ in NHEK cells. There was also a positive finding in lymphocytes for NM-102, NM-103, and NM-104. The micronucleus assay was negative for all TiO$_2$ in other types of cells.

![Micronucleus assay graphs](image)

**Figure 11.** Micronucleus assay in BEAS 2B cells with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from FIOH).
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Figure 12. Micronucleus assay in 16 HBE cells with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from IPL).
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Figure 13. Micronucleus assay in A549 cells with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from RIVM).
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Figure 14. Micronucleus assay in Caco-2 cells with TiO₂ NM-102, NM-103, NM-104, and NM-105 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from ANSES).
Figure 15. Micronucleus assay in primary human lymphocytes with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 30-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from INSA).

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Figure 16. Micronucleus assay in NHEK cells with TiO$_2$ NM-102, NM-103, NM-104, and NM-105 after 48-h treatment. Results from two independent experiments are shown. Symbols show means of duplicate cultures, error bars SE (Data from IMB-BAS).
Mouse lymphoma assay

The results of the mouse lymphoma test with TiO$_2$ are shown in Figs 17 and 18. The mouse lymphoma mutation assay was negative for all forms of TiO$_2$ tested.

**Figure 17.** Mutation frequency (red line) in L5178Y TK +/- cells after treatment with TiO$_2$ NM-102 and NM-103. Symbols show means of duplicate cultures. Results from two experiments (left and right) are shown for each MN. RTG, relative total growth (a measure of cytotoxicity (blue line) (Data from IPL).
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Figure 19. Comet assay in BEAS 2B cells with SAS NM-200, NM-201, NM-202, and NM-203 after 3-h treatment. Symbols show means of medians of duplicate cultures, error bars SE (Data from IPH).
Figure 20. Comet assay in 16 HBE cells with SAS NM-200, NM-201, NM-202, and NM-203 after 3-h and 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from UAB).
Figure 21. Comet assay in A549 cells with SAS NM-200, NM-201, NM-202, and NM-203 after 3-h and 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from IPH).
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Micronucleus assay
Results of the micronucleus assay with SAS in various cell systems are shown in Figs 23-28.
In BEAS 2B cells, NM-200, NM-201 and NM-202 were negative, while NM-203 produced an increase at one dose (equivocal result) (Fig. 23). No micronucleus induction was seen in 16 HBE cells (Fig. 24) or lymphocytes (Fig. 28) by any of the SAS MNs.
In A549 cells two independent experiments were carried out. NM-201 and NM-202 were positive in both of the experiments, while NM-200 was negative. NM-203 was negative in the first experiment but showed an increase in micronuclei at one dose in the second experiment (results considered equivocal).
In undifferentiated Caco-2 cells (52-h treatment), three independent micronucleus assays were performed for each of the SAS MNs (Fig 26). In the first experiment, all of the SAS MNs were positive. In the second series, however, all SAS MNs gave a negative result. In the following repeat, positive results were again recorded for all four SAS MNs. The overall judgement for micronucleus induction in Caco-2 cells by all SAS MNs could be considered positive, but due to the variable results of repeated experiments, the outcome of NM-200, NM-201 and NM-202 was marked with a +/- sign in Table 2 which summarises the findings. In differentiated Caco-2 cells (24-h treatment), none of the SAS MNs was able to induce micronuclei (Fig. 27).

![Graphs](https://via.placeholder.com/150)

**Figure 23.** Micronucleus assay in BEAS 2B cells with SAS NM-200, NM-201, NM-202, and NM-203 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from FIOH).
Figure 24. Micronucleus assay in 16 HBE cells with SAS NM-200, NM-201, NM-202, and NM-203 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from IPL).
Figure 25. Micronucleus assay in A549 cells with SAS NM-200, NM-201, NM-202, and NM-203 after 48-h treatment. Results are shown from two independent experiments. Symbols show means of duplicate cultures, error bars SE (Data from INRS).
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Figure 28. Micronucleus assay in human peripheral lymphocytes with SAS NM-200, NM-201, NM-202, and NM-203 after 30-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from IPL).
**Mouse lymphoma assay**

Results from the mouse lymphoma mutation assay with SAS are shown in Figs 29 and 30.

None of the SAS MNs was able to induce mutations in the mouse lymphoma mutation assay.

**Figure 29.** Mutation frequency (red line) in L5178Y TK +/- cells after treatment with SAS NM-200 and NM-201. Symbols show means of duplicate cultures. Results from two experiments (left and right) are shown for each MN. RTG, relative total growth, a measure of cytotoxicity (blue line) (Data from IPL).
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were also included as a negative control. Exposure was done using MEM medium with 5 % foetal calf serum (FCS).

The following steps were done at the platform. Briefly, after a subsequent step of fixation and post-fixation with glutaraldehyde and osmium tetroxide respectively, cells were dehydrated in ethanol and infiltrated in acetone-eponate and then in pure eponate. Finally, cells were embedded in DMP30-Eponate, cut with a LEICA UC7 microtome and stained with toluidine blue. Ultra-thin sections (90 nm) were collected onto copper grids and counterstained with 4 % uranyl acetate then with lead citrate (Reynold solution). Examination was performed with JEOL 1400 electron microscope operated at 120 kV.

Compared to the negative control (Figure 31), TEM images of treated cells showed that all the SAS were taken up and induced toxicity in Caco-2 cells (Fig. 32) especially NM-202 (Fig. 32c) and -203 (Fig. 32d). In the cells exposed to these MNs, a larger number of vacuoles and myelinic figures were seen compared to NM-200 (Fig. 32a) and NM-201 (Fig. 32b). In the cytoplasm, SAS was mainly localized inside endocytic compartments. In one cell exposed to NM-202, nanoparticles could be detected inside mitochondria (data not shown). Although nuclei were deformed in all the cells, none of the SAS could be detected inside the nucleus.

![Figure 31. TEM image of unexposed Caco-2 cells.](image)

As another supplementary analysis, the release of the cytokine interleukin 8 (IL-8) was measured by an ELISA technique after treatment of Caco-2 cells with the SAS MNs. Undifferentiated and differentiated Caco-2 cells were seeded in 24-well plates at a density of 50 000 cells/cm² and 20 000 cells/cm², respectively. After a 24-h treatment with SAS, cell supernatants were collected, centrifuged at 10 000 x g for 2 min and stored at -20 °C until analysis. IL-8 release was measured with a spectrophotometer plate at a wavelength of 405 nm.

In undifferentiated cells, the SAS treatment did not increase IL-8 release, despite a clear induction of IL-8 was observed in cells treated with NM-202 and NM-203 in a preliminary experiment (data not shown). In differentiated cells incubated with SAS, the release of IL-8 was slightly higher than in
undifferentiated cells, especially in cells treated with NM-203, where the ratio of IL-8 compared with the control was 1.8 and 2.5 at 128 µg/ml and 256 µg/ml, respectively (data not shown).

An increase in IL-8 secretion was detected in undifferentiated Caco-2 cells exposed to MWCNTs NRCWE-006 but not to the TiO₂ MNs (data not shown).

**Figure 32.** TEM images of Caco-2 cells following 24-h exposure to 256 µg/ml of SAS NM-200 (a), NM-201 (b), NM-202 (c) and NM-203 (d). Black arrows indicate internalized SAS.

**MWCNT**

*Comet assay*

Results from the comet assay on MWCNTs are shown in Figs 33-39. The comet assay was negative for all MWCNTs. In BEAS 2B and Caco-2 cells, MNCNTs were also studied using the FpG-modified comet assay - with negative results (data not shown; Tables 1 and 2).
Figure 33. Comet assay in BEAS 2B cells with MWCNTs NM-400, NM-401, and NM-402 after 3-h and 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from UAB).
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Figure 35. Comet assay in 16 HBE cells with MWCNTs NM-400, NM-401, and NM-402 after 3-h and 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from NRCWE).
Figure 36. Comet assay in 16 HBE cells with MWCNTs NM-403, NRCWE-006, and NRCWE-007 after 3-h and 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from NRCWE).
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Figure 38. Comet assay in A549 cells with MWCNTs NM-403, NRCWE-006, and NRCWE-007 after 3-h and 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from NRCWE).
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Figure 39. Comet assay in Caco-2 cells with MWCNTs NM-400, NM-401, and NM-402 after 3-h and 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from UAB).
Figure 40. Comet assay in Caco-2 cells with MWCNTs NM-403, NRCWE-006, and NRCWE-007 after 3-h and 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from UAB).
Micronucleus assay

Results of the micronucleus assay with MWCNTs in the various cell systems are shown in Figs 41-45. The micronucleus assay was mostly positive for MWCNTs in BEAS 2B, A549 (NM-401 and NM-403 negative) and Caco-2 (NRCWE-006 negative) cells, but negative for all MWCNTs in 16 HBE cells. In lymphocytes, MWCNTs NM-403 and NRCWE-006 were positive and NM-402 was equivocal, while the other MWCNTs were negative.

Figure 41. Micronucleus assay in BEAS 2B cells with MWCNTs NM-400, NM-401, NM-402, NM-403, NRCWE-006, and NRCWE-007 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from FIOH).
Figure 42. Micronucleus assay in 16 HBE cells with MWCNTs NM-400, NM-401, NM-402, NM-403, NRCWE-006, and NRCWE-007 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from IPL).
Figure 43. Micronucleus assay in A549 cells with MWCNTs NM-400, NM-401, NM-402, and NM-403 after 48-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from IPH for NM-400 and NM-401, INSA for NM-402 and NM-403, RIVM for NRCWE-006 and NRCWE-007).
Figure 44. Micronucleus assay in Caco-2 cells with MWCNTs NM-400, NM-401, NM-402, and NM-403 after 52-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from ANSES).
Figure 45. Micronucleus assay in human primary lymphocytes with MWCNTs NM-400, NM-401, NM-402, NM-403, NRCWE-006, and NRCWE-007 after 30-h treatment. Symbols show means of duplicate cultures, error bars SE (Data from IPL for NM-400 and NM-401, INSA for NM-402, NM-402, NRCWE-006 and NRCWE-007).

Mouse lymphoma assay

Results from the mouse lymphoma mutation assay with MWCNTs are shown in Fig. 46. The results were negative for all types of MWCNTs.
Figure 46. Mutation frequency (red line) in L5178Y TK +/- cells after treatment with MWCNTs NM-400 and NM-401, NM-402, NM-403, NRCWE-006, NRCWE-007. Symbols show means of duplicate cultures. NRCWE-006 was studied in two independent experiments. RTG, relative total growth, a measure of cytotoxicity (blue line) (Data from IPL).
NM-110 (ZnO)

Comet assay

An example of the comet assay with ZnO NM-110 in three cell lines is shown in Fig. 47. A dose-dependent induction of DNA damage was seen in all cell lines with a 3-h treatment, but BEAS 2B cells appeared to be the most susceptible to ZnO, followed by Caco-2 cells and A549 cells. The comet assay with NM-110 was also positive in NHEK cells (data not shown).

![Comet assay graph](image)

**Figure 47.** Comet assay in BEAS 2B, Caco-2 and A549 cells with ZnO NM-110 after a 3-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE (Data from NIOM).

Micronucleus assay

Studies performed with BEAS 2B cells in the beginning of the project showed a dose-dependent induction of micronuclei by ZnO; fluorescence in situ hybridization with pancentromeric and pantelomeric probes suggested that the micronuclei were formed by both clastogenic and aneugenic mechanisms (data not shown).

Based on these findings, ZnO NM-110 was incorporated as a candidate positive nanoparticle control in the actual series of the first part of WP5. Fig. 48 shows micronucleus induction by ZnO NM-110 in different cell types after being included in the experiments at 1-3 doses. A more pronounced effect was seen in A549 cells and Caco-2 cells than in BEAS 2B cells, NHEK cells or human lymphocytes. In general, human lymphocytes were clearly less sensitive to NM-110 than the various human cell lines.

As both the comet assay and the micronucleus assay suggested that ZnO is genotoxic, NM-110 was also included in the round robin study to further assess its possible use as a nanosized positive control.
Figure 48. Micronucleus assay with ZnO NM-110 in various human cell types after treatment for 1.5-2 cell cycles. Symbols show means of data from different experiments in different laboratories. Error bars show SE. Grey columns represent concurrent control cultures.
Conclusions from the 1st part of WP5

In the 1st part of WP5, data on the genotoxicity of 15 MNs (4 TiO$_2$, 4 SAS, 6 MWCNTs, and ZnO as a candidate positive nanoparticle control) were generated using the comet assay and the micronucleus assay on a number of different human cell systems of pulmonary, intestinal, and epidermal origins. In addition, micronuclei were also studied in human primary lymphocytes, and mutations in mouse lymphoma cells.

Tables 1 and 2 summarize results from the 1st part of WP5. The studies showed that all of the assays are technically feasible for testing the genotoxicity of MNs.

While the mouse lymphoma assay gave uniformly negative results, 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 variation, e.g. in MN dispersion, agglomerate size 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), and variation in scoring.

Although dose-dependent effects could be seen in many experiments, the genotoxicity of the MNs studied was usually relatively low; in such a situation, experimental variation may determine, if the result will turn out positive or negative. This was also suggested by the fact that repeated experiments in the same laboratory did not always result in the same overall outcome (positive or negative). The most extensive studies were carried out using the micronucleus assay with SAS in Caco-2 cells. Although the results were mostly positive, some of the independent series yielded negative results. It is presently unclear whether these findings reflect variation in the solubility of the SAS or other sources of experimental variation. It may be possible that similar fluctuations also concern other MNs.

In general, different types of SAS showed some induction of DNA damage and micronuclei in various cell systems. Exclusively negative results were obtained with SAS MNs in human primary lymphocytes, and in 16 HBE cells the only positive result was induction of DNA damage by NM-200.

Of the various forms of TiO$_2$, NM-102 (anatase) and NM-105 (anatase-rutile) were mostly able to induce DNA damage, in agreement with the expected higher surface activity of anatase and anatase-rutile mixture than pure rutile. No DNA damage induction by the TiO$_2$ MNs was, however, observed in the reconstructed skin model, apparently due to poor penetration. Micronucleus induction by the TiO$_2$ MNs was not seen in most of the cell lines, except for NHEK cells and primary lymphocytes.

MWCNTs did not induce DNA damage, but micronuclei were produced by many of them in different cell systems except 16 HBE cells.
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Table 1. Outcome of *in vitro* genotoxicity assays in pulmonary cell lines (1st part of WP5).

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>BEAS 2B</th>
<th>16 HBE</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Micronucleus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Comet, 3 h</td>
<td>Comet, 24 h</td>
</tr>
<tr>
<td>TiO&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM-102</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NM-103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NM-104</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NM-105</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>SAS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM-200</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>NM-201</td>
<td>-</td>
<td>(-)</td>
<td>+</td>
</tr>
<tr>
<td>NM-202</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NM-203</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MWCNT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM-400</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NM-401</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NM-402</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NM-403</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NRCWE-006</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NRCWE-007</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Treatment for 48 h, Cyt-B added at 6 h.
<sup>b</sup>Treatment for 41 h, no Cyt-B used.
<sup>c</sup>Treatment for 24 h (TiO<sub>2</sub>, NRCWE-006 and NRCWE-007), Cyt-B added at 6 h.

**Outcome:**

+ 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.
Table 2. Outcome of *in vitro* genotoxicity assays in intestinal, lymphatic and epidermal cell systems (1st part of WP5).

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>Intestinal</th>
<th>Lymphatic</th>
<th>Epidermal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caco-2</td>
<td>L5178Y TK+/-</td>
<td>NHEK</td>
</tr>
<tr>
<td></td>
<td>Micronucleus(^a)</td>
<td>Comet, 3 h</td>
<td>Comet, 24 h</td>
</tr>
<tr>
<td>TiO(_2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM-102</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NM-103</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>NM-104</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NM-105</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SAS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM-200</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NM-201</td>
<td>+/-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>NM-202</td>
<td>+/-</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>NM-203</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MWCNT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM-400</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>NM-401</td>
<td>+</td>
<td>-</td>
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<tr>
<td>NM-402</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NM-403</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NRCWE-006</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NRCWE-007</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Treatment for 52 h, Cyt-B added at 24 h.
\(^b\) Treatment for 30 h, Cyt-B added at 6 h.
\(^c\) Treatment for 54 h, Cyt-B added at 6 h.

OUTCOME:
+ , 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;
Grey box, not performed.

\(^1\) Three independent experiments performed by the same laboratory yielded a positive outcome twice and a negative outcome once.
2\textsuperscript{nd} part: round robin test

NM-102 (TiO$_2$)

In BEAS 2B cells, the outcome of the comet assay with NM-102 (Fig. 49), was almost unanimously positive (five out of six laboratories), in accordance with the outcome of the 1\textsuperscript{st} part of WP5. The result of the micronucleus assay in BEAS 2B cells was negative in four laboratories (Fig. 50) - in agreement with the 1\textsuperscript{st} part - positive in one laboratory, and equivocal in another laboratory.
Figure 49. Comet assay in BEAS 2B cells with TiO$_2$ NM-102 after 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE. INSA performed two independent experiments (shown separately).
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Figure 51. Comet assay in Caco-2 cells with TiO₂ NM-102 after 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE. ANSES, NRCWE, and INRS performed two or more independent experiments (shown separately).
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Figure 53. Comet assay in BEAS 2B cells with SAS NM-203 after 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE. INSA performed two independent experiments (shown separately).
Figure 54. Micronucleus assay in BEAS 2B cells with SAS NM-203 after 48-h treatment. Symbols show means of duplicate cultures, error bars SEs (in IPH, duplicates were pooled and SE is not shown). INSA performed two independent experiments (shown separately).

In the comet assay with Caco-2 cells, NM-203 (positive in the 1st part) was negative in three and positive in two laboratories (Fig. 55). The results of the micronucleus assay with NM-203 in Caco-2 cells were split (Fig. 56), with three positives and three negatives; conflicting results had also been obtained in the 1st part.
Figure 55. Comet assay in Caco-2 cells with SAS NM-203 after 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE. ANSES, NRCWE, and INRS performed two or more independent experiments (shown separately).
Figure 56. Micronucleus assay in Caco-2 cells with SAS NM-203 after 48-h treatment. Symbols show means of duplicate cultures, error bars SEs. ANSES and INRS performed two independent experiments (shown separately).
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 (Fig. 57). 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 (Fig. 58).

**Figure 57.** Comet assay in BEAS 2B cells with MWCNT NM-403 after 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE. INSA performed two independent experiments (shown separately).
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Figure 59. Comet assay in Caco-2 cells with MWCNT NM-403 after 24-h treatment. Symbols show mean of medians of duplicate cultures, error bars SE. ANSES, NRCWE, and INRS performed two or more independent experiments (shown separately).
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Figure 60. Micronucleus assay in Caco-2 cells with MWCNT NM-403 after 48-h treatment. Symbols show means of duplicate cultures, error bars SEs. ANSES and INRS performed two independent experiments (shown separately).
NM-110 (ZnO)

NM-110 (ZnO), tested as a candidate positive nanoparticle control, was unanimously positive in the micronucleus assay with Caco-2 cells (Fig. 61) and yielded 3 positives and 3 negatives in BEAS 2B cells (Fig. 62). In the comet assay, 3 negatives and 2 positives were recorded in Caco-2 cells (Fig. 63) and 4 positives and 2 negatives in BEAS 2B cells (Fig. 64).

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

![Caco-2 graph](image)

**Figure 61.** Micronucleus assay in Caco-2 cells with ZnO NM-110 after 48-h treatment. Symbols show means of duplicate cultures, error bars SEs. BFR and IPL performed three independent experiments (shown separately).
Figure 62. Micronucleus assay in BEAS 2B cells with ZnO NM-110 after 48-h treatment as performed by six laboratories. Symbols show means of duplicate cultures, error bars SEs. INSA and IPH performed two independent experiments (separate columns). In four laboratories, the highest dose could not be analysed due to toxicity.

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The outcome of the round robin study is summarized in Table 3. Both the comet assay and the micronucleus assay were chosen as the genotoxicity tests for the round robin study. Human bronchial epithelial BEAS 2B cells and undifferentiated human epithelial colorectal adenocarcinoma Caco-2 cells were selected as the cell lines to be used. In the absence of information on the correctness of the results with respect to MN carcinogenicity or genotoxicity in vivo, these choices were mainly based on practical considerations. One representative of each MN group, NM-102 (TiO$_2$), NM-203 (SAS), and NM-403 (MWCNTs), was chosen to be tested.

In the round robin study, relatively reproducible results were obtained for the comet assay with TiO$_2$ NM-102 in BEAS 2B cells (mostly positive) and with MWCNTs NM-403 in Caco-2 cells (mostly negative), and for the micronucleus assay with MWCNTs NM-403 (mostly negative) in BEAS 2B cells and ZnO 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. The main reason for this appears to be the narrow genotoxic dose range before cytotoxicity. As there is some variation e.g. in dispersions among experiments, the critical dose required for a positive effect is not always reached.

Table 3. Outcome of the round robin test.

<table>
<thead>
<tr>
<th>Partner</th>
<th>TiO$_2$ NM-102</th>
<th>SAS NM-203</th>
<th>MWCNT MN-403</th>
<th>ZnO NM-110</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Comet CBMN</td>
<td>Comet CBMN</td>
<td>Comet CBMN</td>
<td>Comet CBMN</td>
</tr>
<tr>
<td>Caco-2 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANSES</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NRCWE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BfR</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IPL</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RIVM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>INRS</td>
<td>+</td>
<td>-</td>
<td>- (+)</td>
<td>+</td>
</tr>
<tr>
<td>BEAS 2B cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMB-BAS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FIOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NIOH</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UAB</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IPh</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>INSA</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ positive; - negative; (+) equivocal; Grey box, no data; $^1$ In 3-h treatment - 24-h treatment was negative; $^2$ In 24-h treatment - 3-h treatment was negative.
Taken together, 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. With a few exceptions (e.g. ZnO in some systems), 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. Comparison of data obtained from WP5 and WP6 suggested that while some in vivo findings agreed with the in vitro outcome, examples of apparent in vivo genotoxicity that was not detected in vitro were found. As some of the positive in vivo findings appeared to be systemic, these cases probably represented secondary genotoxicity which may be very difficult to detect in vitro. The predictive value of in vitro tests in identifying MNs that are genotoxic in vivo can better be clarified, if more information is obtained on the mechanisms of (i) the detected in vitro genotoxicity and (ii) the MNs that are genotoxic in vivo or carcinogenic. These issues are further discussed in the next chapter.

In vitro genotoxicity testing strategy for MNs: recommendations for further studies

Genotoxicity testing of MNs - general considerations about genotoxic carcinogens

The ultimate goal of WP5 was to formulate a strategy for genotoxicity testing of MNs, based on the results obtained in the 1st and 2nd part of the workpackage and data available from the literature and other sources such as different research projects.

In the round robin phase, WP5 concentrated on two genotoxicity endpoints (DNA damage by the comet assay and chromosome alterations by the micronucleus assay) and two cell lines, virus-transformed bronchial epithelial BEAS 2B cells and heterogeneous human epithelial colorectal adenocarcinoma Caco-2 cells. The general conclusion was that technically both genotoxicity endpoints and cells lines are applicable to in vitro genotoxicity testing of MNs. Nevertheless, our study should not be viewed as a validation exercise for the two endpoints and cell lines. Yet, the techniques and protocols developed and the experience gained in NANOGENOTOX are expected to be useful for scientists and regulators in the field. Our studies were designed and conducted using the best current understanding of these issues, although they may not represent the final word on how genotoxicity testing of MNs is to be conducted in the future.

Strategy can be defined as a high-level plan to achieve one or more goals under conditions of uncertainty. The primary goal of an in vitro genotoxicity testing of MNs is to identify MNs that can be carcinogenic due to their genotoxic properties. In principle, genotoxicity assays are able to detect carcinogens that mainly act via genotoxic mechanisms. By definition, this goal leaves out the detection of carcinogenic MNs that have another mode of action than genotoxicity.

Uncertainty is the key word in the present situation with genotoxicity assessment of MNs. First of all, there are carcinogenicity data on very few MNs. This means that in the case of MNs we cannot yet follow the usual practice applied in validating short-term assays for genotoxic carcinogens, which includes testing panels of known carcinogens and non-carcinogens and assessing such parameters as the specificity or sensitivity of the assay in correctly identifying the substances in the two panels. Furthermore, it is unclear how important genotoxic events actually are in the carcinogenesis of the MNs that have been shown to be carcinogenic.
Nanosized TiO$_2$ anatase-rutile (similar to NM-105 studied in NANOGENOTOX - other TiO$_2$ MNs have not been studied for carcinogenicity), fine TiO$_2$, and carbon black have been described to induce lung cancer in rats after intratracheal instillation or inhalation exposure. Although genotoxicity has been considered to play some role in the carcinogenesis of TiO$_2$ and carbon black and other small particles with no specific toxicity, the genotoxic mechanism has usually been assumed to be secondary. If this is true, the carcinogenic action of nanosized TiO$_2$ and carbon black could have a threshold dose. In reality, however, very little is known about the carcinogenic process of MNs. With TiO$_2$, there is some indication that nanosized TiO$_2$ is carcinogenic at lower doses than fine TiO$_2$. If some MNs were carcinogenic at substantially lower exposure levels than larger particles, they might pose a considerable cancer risk even if their mode of action would involve secondary genotoxicity.

Long, rigid MWCNTs have been shown to induce mesothelioma when injected intraperitoneally or intrascrotally to mice and rats, and thus appear to conform to the fiber paradigm and resemble asbestos. Genotoxicity has been advocated to have a role also in MWCNT carcinogenesis, but again the question is whether the effect is secondary or primary (but indirect).

It is presently obvious that the existing information on carcinogenicity cannot be used in evaluating the performance of genotoxicity tests in identifying carcinogenic MNs - before more MNs have been tested for carcinogenicity and before more light is shed on the mechanisms involved. Meanwhile, we have to rely on expert judgment in interpreting the outcome of the genotoxicity assays.

**Comparison of MN genotoxicity in vitro and in vivo**

It currently appears to be a justified guess that bio-persistent MNs that are able to induce a prolonged genotoxic effect in vivo, locally or systemically, may be carcinogenic. However, there are presently all too few in vivo studies that have examined genotoxic effects in target tissues, and studies with extended observation period are almost non-existent. When information of MN carcinogenicity and modes of action is missing, comparative results from relevant in vivo tests are in a key position when considering the relevance of in vitro genotoxicity assays.

NANOGENOTOX is one of the few projects that have provided data on the genotoxicity of exactly the same MNs in vitro and in vivo. It is particularly valuable that WP6 also included the micronucleus assay in some target tissues, in addition to the comet assay. The comet assay is a flexible tool in vivo and can be applied to various tissues, but as it depicts transient DNA damage most of which will be repaired, it may be necessary to follow up the persistency of the DNA damage - to see if DNA damage is continuously produced or present still a long time after the end of the exposure.

Conversely, the micronucleus assay is an indicator of DNA lesions (and aneugenic effects) that were not repaired correctly and, therefore, a measure of damage that cannot anymore be corrected. Induction of micronuclei even after a short follow-up time is a more serious sign of a hazard than transient DNA damage. Hence, there is an urgent need to further develop micronucleus techniques (or other indicators of chromosome damage) for target tissues of particle carcinogenesis, especially lung epithelium. Information on in vivo assays for gene mutations, another irreversible endpoint, would likewise be needed, although such techniques have only rarely been applied in studies of MNs; besides the LacZ assay used in WP6, several systems exist for this purpose, including other transgenic mouse models, the traditional Hprt assay, and (at least for a systemic effect) the more recently developed Pig-A test.

In the following, the various genotoxicity tests applied in WP5 are considered in general and in connection with in vivo results from WP6.
Considerations on the comet assay and other assays of DNA damage in vitro

The comet assay is an excellent research tool in assessing the ability of various substances to induce transient DNA damage. As the DNA damage detected by the comet assay is subject to DNA repair, the method can be used to show persistent or continuously produced DNA damage - and the disappearance ("repair") of the damage. Many in vitro studies have suggested that biopersistent MNs tend to produce DNA damage days after the start of the exposure. It is not, however, known how long such DNA damage could prevail in vitro. In WPs, the 24-h continuous treatment used was also often associated with an increased level of DNA damage. Although the increase in DNA damage associated with MNs of low specific toxicity is relatively small and may not be reproducible from one experiment to the other, it would be important to understand, how and at which doses this DNA damage is formed. Is it mostly a high-dose effect associated with, e.g., oxidative stress or is there an initial increase at low doses which then levels or even turns down at higher doses? There seemed to be examples of both types of dose-responses in the present workpackage. Nevertheless, DNA damage at low doses in vitro has not systematically been examined with MNs.

The FpG-modified comet assay was used by some partners as an additional test for SAS in BEAS 2B, 16 HBE, A549 and Caco-2 cells and for MWCNTs in BEAS 2B and Caco-2 cells. This approach was supplementary and was not available for all MNs and cell systems. However, it did not seem to reveal much new information. It may be that the proportion of oxidative DNA damage detectable by the FpG modification was not high enough for the MNs and cell systems studied. It has also been suggested that the MN present on the slides during the enzyme treatment could hamper the enzyme reaction, but further studies are needed to see if this could be the case with some MNs. As oxidative stress is assumed to be generally important for many MNs, more information is needed on the correlation of this phenomenon with genotoxicity and on the formation of oxidative DNA damage, also by using applicable techniques for the analysis of oxidative DNA adducts.

Ultimately, the questions are, how well the in vitro comet assay reflects possibility for DNA damage in vivo and how similar the mechanisms of MN genotoxicity operating in vitro and in vivo are. The main issue is, whether inflammation, which is not fully available in the present in vitro systems, is a major determinant of DNA damage in vivo.

Comparison of comet assay results obtained in WP5 and WP6 is hampered by missing in vivo data and the low and variable genotoxic activity of the MNs, so that each MN could not clearly be defined as positive or negative.

- Among TiO₂ MNs, NM-102 and NM-105 were more often positive than NM-103 and NM-104 in the comet assay in vitro, and NM-105 seemed to be the only TiO₂ MN that could increase DNA damage in bronchoalveolar lavage (BAL) cells of rats in vivo after instillation. However, after exposure by gavage, other TiO₂ MNs seemed to increase DNA damage in the intestine (NM-103) or colon (NM-102, NM-104) but also in spleen (NM-103) and bone marrow (NM-104), suggesting systemic effects. In vitro, NM-103 and NM-104 showed only an equivocal increase in DNA damage in NHEK cells and (NM-103) in Caco-2 cells.

- SAS MNs, which showed DNA damage induction in vitro, appeared to be negative in the comet assay in vivo. The only in vitro approach that predicted this result was comet assay in 16HBE cells after 24-h treatment.
In vitro, MWCNTs did not induce DNA damage in the 1st phase of WP5 (although some positive comet assay results were obtained with NM-403 in the round robin study). However, the in vitro comet assay indicated an increase in DNA damage in kidneys after instillation of NM-400, NRCWE-006 (equivocal), NM-401 (with FpG), and NM-402 (with FpG) and gavage of NM-401 and NM-400 (equivocal; with FpG). Local DNA damage was seen after instillation of NM-401 in BAL cells and of NRCWE-006 (equivocal effects) in BAL cells and lungs with NM-401; in addition equivocal systemic DNA damage was detected in the spleen after instillation of NM-401 (with and without FpG). Thus, it appears that most of the in vitro comet assays were unable to predict these in vivo effects which might have represented secondary genotoxicity.

The in vitro comet assay is presently not among the regulatory genotoxicity assays for chemicals, and it may, therefore, be unlikely that it will be used for this purpose with MNs, either. The transient nature of the DNA damage detected by the comet assay and the primarily positive responses obtained in this assay with many MNs are often seen as a problem. In the present data, however, many MNs did not induce DNA damage detectable by the comet assay. It is yet premature to say whether the in vitro comet assay, with some modifications, could be used to show, e.g., persistent DNA damage occurring at low doses.

Considerations on the micronucleus assay and other assays of chromosome damage in vitro

Micronuclei are formed by both misrepaired (or unrepaired) DNA damage (clastogenic effect) and missegregation of chromosomes (aneugenic effect) and, therefore, reflect a wide spectrum of non-reversible genotoxic phenomena. Nevertheless, the mode of action of MNs that are able to increase micronuclei is usually not known. This could be examined by, e.g., using centromeric and telomeric FISH, as was done for ZnO in the present study. There is presently no consensus on the timing to be used when testing micronucleus induction by MNs. In the present studies, we used a continuous treatment for 1.5-2 cell cycles, but also shorter and longer treatment times have elsewhere been applied. We added Cyt-B at 6 h (in some cases at 24 h) after the start of the MN treatment, in accordance with the idea that Cyt-B added simultaneously with the MN may prevent endocytosis of the MN. It has not systematically been studied, which timing is optimal for MNs. It may be of interest that all MNs were negative in 16 HBE cells where Cyt-B was not used; this finding could reflect a general insensitivity of this cell line to micronucleus induction by MNs, but it could also be due to the fact that the low capacity of some MNs to induce micronuclei is difficult to show, unless the analysis exclusively concerns cells that have divided once after MN addition; in the cytokinesis block technique, this means binucleate cells. It may generally be recommended that, when the in vitro micronucleus assay is used for MNs, the cytokinesis block method is applied - although it may be necessary to examine more closely what the optimum time of Cyt-B addition actually is.

Interestingly, some induction of micronuclei by TiO₂ and MWCNTs was seen in cultured human lymphocytes, in agreement with some earlier studies. As it is likely that lymphocytes do not actively take up MNs, this finding may suggest that the genotoxic effects of MNs in lymphocytes are indirect, mediated, e.g., by the influence of the MNs on lymphocyte membrane, by the monocytes (capable of phagocytosis) present in the cultures, or by an unspecific effect of MNs on culture conditions. Poor uptake to lymphocytes might also explain the marginal effect on micronucleus frequency of the partly soluble ZnO which was clearly active for instance in Caco-2 cells. It might be worth the effort to look at MN behaviour in the lymphocyte system more closely, as the suspected indirect genotoxic
effect could concern other types of cells as well. On the other hand, a cell type that shows poor uptake of MNs may not be recommended for genotoxicity testing of MNs.

- In WP6, data from the micronucleus assay was partly missing, but the findings available were negative with the exception of intravenous treatment with SAS NM-203 where an increase in micronuclei was seen in bone marrow after the highest dose; this response agreed with the in vitro micronucleus assay in two of three experiments with Caco-2 cells, one of two experiments in A549 cells (equivocal), and one experiment in BEAS 2B cells (equivocal) in the 1st part of WP5. Half of the laboratories got a positive result in the micronucleus assay with NM-203 in the round robin study with both BEAS 2B cells and Caco-2 cells. Caco-2 cells gave a positive result in two out of three experiments for the rest of the SAS as well, and NM-201 and NM-202 were also positive in A549 cells.

- In the 1st part of WP5, most micronucleus assays with TiO$_2$ MNs were negative, in agreement with the in vivo outcome. However, NHEK cells showed micronucleus induction for all TiO$_2$ MNs and human lymphocytes for NM-103, NM-104, and (equivocal) NN-102, disagreeing with the in vivo data.

- Although MWCNTs induced micronuclei, with a few exceptions, in BEAS 2B cells, A549 cells, and Caco-2 cells in vitro, the in vivo micronucleus data available for MWCNTs in WP6 were negative, in accordance with results obtained with 16 HBE cells in vitro. Also with soluble chemicals, a positive in vitro micronucleus assay is not always reproduced in vivo, reflecting the fact that in vitro assays are usually more sensitive than in vivo assays. Depending on the exposure route, MNs may not often reach the tissue examined for micronuclei; this especially appears to be true for the bone marrow micronucleus test.

The in vitro micronucleus assay is presently much used for testing of chemicals and has often been advocated as the method of choice for testing of MNs as well, as it depicts damage that is not repairable. To justify this view, more comparative data are, however, needed from in vivo studies especially from target tissues. Like with the comet assay, it should be examined if the mechanisms that result in an increase in micronucleus frequency are similar in vitro and in vivo and if inflammation plays an important part in micronucleus formation in vivo. The micronucleus test is usually considered to produce less positive results in vitro with MNs than the comet assay, but this did not seem to be the case for the MNs studied in WP5.

**Considerations on the mouse lymphoma assay in vitro**

Besides the Ames test in bacteria, gene mutation assays have rarely been used for in vitro testing of MNs. It seems to be a general experience that the Ames test is not suitable for genotoxicity testing of MNs, mainly because of the poor uptake of MNs by the bacteria. Yet, gene mutations are an important class of irreversible genotoxic effects which have a well-established role in carcinogenesis. Thus, it would be important to cover this endpoint, too.

- In WP5, all MNs were negative in the mouse lymphoma mutation assay, in agreement with most of the limited mutation studies performed in vivo with the LacZ assay. SAS NM-202 and NM-203 were an exception and showed mutation induction at a low dose in colon cells. This finding may represent secondary genotoxicity. It also has to be remembered that the mutational endpoints were not necessarily directly comparable in vitro and in vivo. While the LacZ test is primarily a gene mutation assay, the mouse lymphoma assay depicts a wide variety of mutational events.
Mammalian gene mutation assays are usually relatively insensitive. Insensitivity to MNs of low genotoxicity may also explain the exclusively negative result obtained with all MNs in the mouse lymphoma assay which reflects various types of mutations including gene mutations and recombination. As we do not know which outcome would be correct for the MNs studied with respect to genotoxic carcinogenesis and in vivo induction of mutations, we need to wait for further studies on MNs in mutagenicity assays. In this context, studies involving prolonged treatments may be of interest.

**Considerations on in vitro exposure of cells to MNs and cellular uptake of MNs**

The need to prepare stable dispersions on MNs before they are tested in vitro is a basic prerequisite for a successful genotoxicity assay. If the dispersion is not adequate, the doses delivered to the cultures may not be what they are intended to be. In NANOGENOTOX, a standardized technique for preparing MN dispersions was used WP5 and other WPs. As explained in WP4, the technique was universal but suited better to some MNs than others. The key issues in the dispersion technique were pre-wetting with ethanol and the use of a small amount of bovine serum albumin (BSA) to aid the dispersion. The use of BSA has raised a lot of discussion, as the combination of BSA and sonication seems to produce particles firmly covered with BSA. This may alter the surface properties of the MNs and thereby possibly cellular uptake and outcome of the genotoxicity test. On the other hand, all cell culture media contain protein, and the high proportion of bovine serum (rich in albumin) used in most systems will probably anyway coat the MNs with albumin. Also in vivo, MNs entering the body are covered with proteins, although the proteins involved for instance in the lungs are different than in blood circulation. Nevertheless, the technique of MN dispersion remains an important question in testing the genotoxicity of MNs. Alternative ways of performing the in vitro exposure, e.g., by air-liquid interface systems, are less explored but should also be studied. It should, however, be kept in mind that liquid-interface systems are presently in a developing stage and their application to in vitro toxicity testing is a demanding task requiring specific expertise, e.g., in aerosol physics.

Some of the variation in assay outcome experienced in WP5 may have derived from variable success in preparing the MN dispersions. Even if the MN dispersion is agreeable when added to the culture, further dispersion to the culture medium determines how much and to which size of agglomerates the cells are exposed to. If the dispersion is inferior, the MN quickly sediments to the bottom, and cells growing on surface contact a high amount of large agglomerates which may or (if too large) may not be taken up by the cells. A perfect dispersion resembles a solution: cells on the bottom of the culture vessel deal with relatively few small agglomerates and take up them but will not get a high intracellular dose. The behaviour of the dispersion in growth medium may change with the dose of the MN added to the culture. It is easy to imagine that differential sedimentation may significantly affect the outcome of the genotoxicity assay, and is, therefore, one of the important questions to be studied further when using in vitro systems.

Although detailed studies are not available, it can be assumed that the cellular uptake of MNs and intracellular dose influence the outcome of an in vitro genotoxicity assay. Various techniques to show or quantify uptake are available, including electron microscopy, fluorescence microscopy (MN with fluorescent label), hyperspectral microscopy, and flow cytometry. If intracellular dose could reliably be determined, it could provide a means to compare responses among different cell systems in vitro and in vivo and among various experimental conditions.
Recommendations for future research and testing

A number of topics that could and should be studied in future studies aiming at developing genotoxicity testing of MNs were considered above. An optimum evaluation of genotoxicity test performance would require that the model MNs used in this task can be classified with respect to their genotoxic carcinogenicity or lack of it. While further carcinogenicity data on MNs are urgently needed, it will take a while before they will be available. Meanwhile, an educated choice is to further examine correlations between in vitro and in vivo genotoxicity of MN, to devise in vitro assays that could adequately predict genotoxic activity of MN in vivo.

It would also be essential to carry out studies that elucidate (a) the mechanisms of action (and role of genotoxic phenomena) of carcinogenic MNs, (b) the genotoxic mechanisms of MNs in vitro and in vivo (and the possible similarity or dissimilarity of these mechanisms), (c) the associations between genotoxicity, inflammation and oxidative stress, (d) the significance of persistent or continuous genotoxic insult possibly induced by MNs in vitro and in vivo, and (e) means of determining intracellular dose. It seems that the toxicological research of MNs is presently full of such fundamental questions that need to be answered, before regulation can be based on proven knowledge.

The chapters above also take up multiple open questions that concern the assays used to detect induction of DNA damage, chromosome damage and mutations by MNs and ways to expose cells (and experimental animals) to MNs. There is still a lot to be learned there, before we can use the methods in similar confidence as they are applied in studies of soluble chemicals.

Some general notions and principles applicable to in vitro genotoxicity testing of MNs can be listed based on WPS findings, the relatively small amount of relevant information available, and on educated guessing:

- Use genetically stable mammalian cells that are able to take up MNs
- BEAS 2B cells seemed to be more practicable and less variable in genotoxicity testing than Caco-2 cells, but both were suitable for this purpose
- Many of the other cell lines applied in WPS also appeared applicable
- Full thickness 3D skin models may not be practical for NM genotoxicity testing (but: suitable for studying NM skin penetration)
- Consider the possible mode of action of the MN
- Partly soluble MNs may act via a Trojan horse effect: culture conditions such as medium may affect test outcome with partly soluble MNs: ratio of dispersion in medium vs dispersion inside the cell
- Many MNs show slight genotoxic activity in vitro, possibly due to indirect mechanisms not yet fully understood; does this mean all such MNs are hazardous?
- Biopersistent MNs may have a continuous genotoxic effects; even low production of DNA damage may be important if it occurs production of seem
- MNs that are genotoxic at low doses are probably more hazardous than MN that become genotoxic only at high doses
• Dose-responses may not be monotonous - there is often an increase in genotoxicity at low doses that is levelled at higher doses; reasons for such low-dose effects should be examined

• As the genotoxic effect of MNs with low specific toxicity effect is slight, it is not easily reproducible - repeat experiments may yield another outcome than the initial experiment

• Success in preparing dispersion is expected to affect agglomerate size, sedimentation, cell exposure, cytotoxicity, choice of doses, and genotoxicity

• Cellular uptake could possibly serve as a measure of dose for comparison among experiments and test systems - techniques should be developed to allow this

• Defining dose range to be tested is primarily based on cytotoxicity determination; however, remember that different assays to evaluate cytotoxicity may result in differential estimates of cytotoxic doses

• It remains to be determined whether testing should actually be stopped at doses
  - that no longer show an increase in cellular uptake
  - where cytotoxicity levels (due to saturation of NM uptake or increase of agglomerate size so that uptake is compromised)

• MNs of low toxicity: how to distinguish real cytotoxicity from secondary toxicity related to, eg, NM compromising culture conditions?

• The possible Influence of dispersant (e.g. BSA) or test outcome is still an unclear issue.
Annexe 1: HaCaT keratinocytes
The WP5 activities of the reporting period concerned specific objective No. 3: To generate in vitro genotoxicity data on NMs and producing in vitro genotoxicity data on the chosen NMs using standard tests and modified assays utilizing specific cell models.

**Partner: IMB-BAS**

1. Cells
The spontaneously immortalized human HaCaT keratinocyte cell line were purchased from German Cancer Research Center, Heidelberg, Germany and were maintained in a 70 % monolayer culture in a Dulbecco’s Modified Eagle’s Medium (DMEM-F12; Lonza) supplemented with 10 % fetal bovine serum (FBS; Lonza). Both cell lines were maintained in a humidified atmosphere containing 5 ± 1% CO₂ in air (standard culture conditions) at 37ºC. The cytotoxicity of the MNs were assessed following 24 h treatment and staining with Trypan blue using a Countess Automated cell counter (Invitrogen).

2. Dispersion of nanomaterials
The preparation of the stock solutions of MNs was achieved following the protocol developed in WP4 (Figure 1).

![Image](image.png)

**Figure 1.** Procedure for MNs preparation
3. Dynamic Light Scattering (DLS)
For all MNs studied (NM102, 103, 104, and 105) a DLS was used to evaluate the dispersion quality. The particle size distribution was determined at 633 nm and 90o with a Dynamic Laser Scatter Bi-90plus equipped with a correlator and avalanche photo detector (APD) (Brookhaven Instruments Corporation, US). Data were analyzed using the BIC Particle sizing Software, vs. 5.23. Samples were measured at 37°C in 4 mL disposable polystyrene cuvettes following different dilutions in complete cell cultured media (DMEM-F12 with 10% FBS). For calculations of hydrodynamic size, a refractive index (RI) of 2.20 for TiO₂ and standard properties for H₂O and solvents were used. The measurements were performed in triplicate (one run consists of three cycles of 3 min).

4. Comet assay
The alkaline version of the Comet assay was performed following the standardized protocol accepted from WP5 members (Figure 2). At the final stage, the slides were washed twice with ultrapure water for 5 min and dehydrated for 10 min into 96% ethanol before being dried at 45°C and stored in the dark at room temperature. For scoring, the slides were stained with 4’6-diamidine-2-phenylindol dihydrochloride (DAPI, 1 µg/ml). Pictures were taken with a fluorescence microscope (Axiovert 200M, Zeiss) and the analyses were performed with TriTek CometScore™ software (TriTek Corporation). A minimum of 100 nucleoids per slide were scored and the percentage of DNA in the tail was recorded.

![Figure 2. Scheme for treatment of normal human epidermal keratinocytes (NHEK) and immortal human keratinocytes (HaCaT) cells.](image)

5. Micronucleus assay
Micronucleus assay was performed following OECD 487 protocols with application of Cytochalasin B in accordance to the scheme shown on Figure 3. The slides were stained with acridine orange and pictured with Axiovert 200M (Zeiss). Nuclei in close proximity with similar staining intensity, staining pattern (distribution of eu- and hetero-chromatin), and approximately of the same size were considered as originating from 1 cell and therefore scored.
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**Figure 3.** Scheme for treatment of normal human epidermal keratinocytes (NHEK) and immortal human keratinocytes (HaCaT) cells.

6. **Detection of DNA fragmentation with Click-iT assay**
The assays were performed following the manufacturer’s instructions (Life technologies, Invitrogen). The cells labelled with Click-iT® TUNEL Alexa Fluor® 488 were counted with FACS Calibur (Becton Dickinson, USA) and analysed with FlowJo software.

7. **Statistical analysis**
The results from COMET assay were analysed by multi-group comparisons of the means of medians carried out by Mann–Whitney U test followed by Jonckheere-Terpstra trend test (PASW Statistics 18.0, IBM, USA). The statistical significance for all tests was set at $p < 0.05$. 
RESULTS

Dynamic light scattering

Following the dispersion of MNs in complete DMEM media, a DLS at 37 °C was performed to characterize the MNs dispersion. It is of great importance in order to ensure that the particles are homogenous in diameter and that there are no aggregates present. Figure 4 shows the intensity particle size distribution (green bars) obtained for NM 102, 103, 104, and 105 at 2 different concentrations (64 and 25 µg/ml) TiO$_2$ used for HaCaT treatment. The data shown are the average of 3 consecutive measurements and are presented as the relative percentage of light scattered by particles (on the Y-axis) in various size classes (on the X-axis). Away of NM102 (64 µg/ml), the size distribution obtained is a bimodal with peak means over a 100 nm. The intensity size distribution obtained implies that there are significant aggregates present in all samples. The conversion of the results into a number based size distribution (Figure 4, orange bars) shows again a bimodal distribution with an average size over 70 nm and with aggregates present up to 1 µm in cell culture media.
Figure 4. Intensity and number distribution of TiO$_2$ nanomaterial suspensions in DMEM-F12 media with 10% FBS measured at 37°C. Mean of 3 independent measurements in triplicates (one run consists of three cycles of 3 min). The results for polydispersity (Pd) are shown as mean±SE.
Toxicity
Following the 24 h treatment with different MNs the toxicity was evaluated by Trypan blue and calculated by Sigmoidal fitting. The results showed a cytotoxic effect of all MNs studied on HaCaT cells (Figure 5) with EC$_{50}$ shown in Table 1.

Figure 5. Cytotoxicity of Titanium dioxides (NM 102, 103, 104, 105) and Zinc oxide (NM 110) nanomaterials in HaCaT cells after 24 h exposure.

Table 1. Estimated EC$_{50}$ following 24h treatment of human keratinocytes HaCaT cells with indicated manufactured nanomaterials

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>EC$_{50}$ [µg/ml]</th>
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<tr>
<td></td>
<td>HaCaT cells</td>
</tr>
<tr>
<td>102</td>
<td>25</td>
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<td>103</td>
<td>17</td>
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<td>120</td>
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<td>105</td>
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COMET Assay
Following the determination of the EC$_{50}$ Comet Assays were performed after exposure to equitoxic EC$_{50}$ concentrations for TiO$_2$ and ZnO nanomaterials. The results are presented in Figure 6 and showed a significant increase in the present of DNA in the tail for NM 103, 104, 105, and 110 following 3h exposure and for NM 102, and 105 at 24 h exposure.

![Alkaline Comets Assay](image)

Figure 6. Alkaline Comets Assay. Mean DNA damage (% DNA in tail) after exposure to equitoxic EC$_{50}$ TiO$_2$ nanomaterials in HaCaT cells following 3 or 24h exposure. n=1000. Error bars show SD. *p< 0.05 compare to the control.

Detection of DNA fragmentation (apoptosis)
To confirm the DNA fragmentation caused by apoptotic mechanisms in HaCaT cells a Click-iT assay was implied. The results showed an increase of the DNA fragmentation following 3 or 24h treatment with different nanomaterials.

3 h

![3h, HaCaT](image)

24h

![24h, HaCaT](image)
Figure 7. Detection of fragmented DNA by FACS in HaCaT cells following treatment with different concentrations of MNs. FL-1H: Channel with green fluorescence indicating the apoptotic fragmented DNA.
Micronucleus assay
All titanium dioxide nanomaterials induced micronuclei in the HaCaT cell line to different extent (Figure 8). NM 102, 104, and 105 showed a dose dependent significant increase (p<0.05) in micronucleated binucleate cells compared to control one.

Figure 8. Micronucleus induction by titanium dioxides in HaCaT cells. Error bars show SD. The positive control treatment (MMC) showed a clear induction of micronuclei (data not shown). N=5, p<0.05.

Conclusions:
1. The dispersion of MNs studded in cell culture media showed presence of aggregates within 10 min of preparation at 37 °C.
2. The TiO₂ nanomaterials cause a cytotoxic and genotoxic effects in HaCaT cells

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Annexe 2: Evaluate the dispersion quality of nanomaterials studied in cell cultured media

1. DLS analysis
For all MNs studied a DLS was used for evaluation of the dispersion quality. The particle size distribution was determined at 633 nm and 90° with a Dynamic Laser Scatter Bi-90plus equipped with a correlator and avalanche photo detector (Brookhaven Instruments Corporation, US). Data were analyzed using the BIC Particle sizing Software, vs. 5.23. Samples were measured at 37°C in 4 mL disposable polystyrene cuvettes following different dilutions in complete cell cultured media (KBM-Gold for NHEK and DMEM-F12 for HaCaT). For calculations of hydrodynamic size, a refractive index (Ri) of 2.20 for TiO₂ and standard properties for H₂O and solvents were used. The measurements were performed in triplicate (one run consists of three cycles of 3 min).

2. Cells
Two different human keratinocytes cell lines were studied: Normal human epidermal keratinocytes (NHEK), growing in complete KGM-Gold media (Lonza) without calcium supplementation and spontaneously immortalized human HaCaT keratinocyte cell line maintained in a Dulbecco’s Modified Eagle’s Medium (DMEM, Lonza) supplemented with 10 % fetal bovine serum (FBS; Lonza).

Results:
The results presented in Figure 1 and 2 showed formations of aggregates in cell culture media studied within 10 min of preparation at 37 °C. Monomodal distribution was recorded for NM 105 in KBM-Gold media and NM110 in DMEM-F12. All other MNs showed a bimodal distribution. The concentrations shown correspond to the one used for treatment of NKEK cells and indicated in the WP5 database.
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Figure 2. Intensity and number distribution of NM110 (4.5 µg/ml) suspensions in complete keratinocytes media (KBM-Gold) and DMEM-F12 with 10% FBS measured at 37°C. Mean of 3 independent measurements in triplicates (one run consists of three cycles of 3 min). The results for polydispersity (Pd) are shown as mean±SE.
Annexe 3: Databases

WP5 generated two databases as part of this deliverable:

Database from the 1st part of WP5 (data generation), containing genotoxicity test results
- for 14 MN (4 TiO$_2$, 4 SAS, 6 MWCNTs), and the nanoparticle control ZnO
- on human pulmonary cell lines (bronchial epithelial BEAS 2B and 16 HBE; alveolar A549), intestinal cell line Caco-2 (primarily undifferentiated cells) and epidermal cell systems (cell line NHEK and 3-D reconstructed full thickness human skin model).
- in the comet assay, the FpG-modified comet assay (for part of the materials and cell systems), the micronucleus assay, and the mouse lymphoma (mutation) assay.

Database from the 2nd part of WP5 (round robin study), containing genotoxicity test results
- for NM-102 (TiO$_2$), NM-203 (SAS), NM-403 (MWCNT), and the nanoparticle control NM-110 (ZnO)
- on BEAS 2B and Caco-2 cells
- in the comet assay and the cytokinesis-block micronucleus assay.

The databases are Annexes to this Deliverable.
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