A novel comprehensive evaluation platform to assess nanoparticle toxicity in vitro

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Abstract. The amount of engineered nanomaterials (ENM) is constantly increasing. Their unique properties, compared to their bulk counterparts, render them suitable for various applications in many areas of life. Hence, nanomaterials appear in a variety of different consumer products leading to the exposure of human beings and the environment during their lifecycle. Even though results on biological effects of ENM are available, harmonized and validated test systems are still missing. One major problem concerning the reliable and robust toxicity testing arises from interactions of ENM with different assay systems. Modifications or damage to DNA can have fatal consequences, such as the formation of tumor cells and hence carcinogenesis. Therefore we focused on the re-evaluation of two genotoxicity assays concerning their nanomaterial compatibility; namely the cytokinesis-block micronucleus cytome assay (MN-assay) and the alkaline single cell gel electrophoresis assay (comet assay). We demonstrate the interference of ENM agglomerates with the read-out of both assays and discuss possibilities how to acquire relevant genotoxicity data.

1. Introduction

Engineered nanomaterials (ENM) possess new properties compared to their bulk counterparts which render them suitable for different applications in many areas of life. Hence, ENM already appear in a huge variety of consumer products and other applications. High expectations from industry as well as science accelerate their production quantity and rate. Consequently, the exposure of human beings and the environment to ENM during their life cycle is inevitable [1]. At the same time concerns arise among consumers concerning the safety aspects of this new technology. As currently toxicological evaluation standards are mostly missing, manufacturers and enterprises face the problem of how to ensure work place safety as well as the safety of their end-products.

Thus two important questions arise: “How to control for the safety of ENM?” or “How to analyze their potential toxicity?”

Even though many studies and results on biological effects of ENM are available, there is still a lack of harmonized and validated test systems. ENM, again due to their exciting special properties, often interfere with assay systems, making reliable conclusions questionable [2-7]. Furthermore, inappropriate suspending methods often lead to false results [8]. Summarizing and comparing all available data to obtain an overall evaluation of potential nano-related toxicity is thus virtually impossible. Therefore, harmonized and robust methods which create a platform for comprehensively validated tools to assess toxicological effects of nanomaterials are urgently needed.
The novel in vitro evaluation platform, we are currently establishing, is based on already existing ISO and OECD guidelines, addressing four key aspects of cytotoxicity, namely the viability of cells, inflammatory reactions, genotoxicity and oxidative stress. Ultimately it’s supposed to offer a fast, reliable, robust, reproducible and sensitive screening tool to detect suspicious nanomaterials. To this end the suitability of at least two independent methods for each of the four parameters will be carefully reassessed and validated. Furthermore a thorough characterization of the applied nanomaterial samples will be included. The reliability and robustness of the assays is subsequently verified by interlaboratory comparisons. A successive and integrated use of this platform will allow reducing the currently prevailing ambiguity concerning the toxicity of nanoparticle containing products. This is fundamental not only for consumers but also for authorities and industry.

Special emphasis during the initial establishment period is laid on detecting interference of ENM with the assay systems themselves and the careful implementation of suitable control experiments. In particular two assays to detect DNA damage turned out to be highly susceptible for disturbance by certain nanomaterials. DNA damage is considered to be a very important parameter to be tested. This type of toxicologic effect might not be directly detectable by simple viability assays as cells are able to repair defective DNA to a certain degree and appear to be healthy for a considerable amount of time. However, accumulation of DNA lesions may later on lead to severe consequences such as cancer and/or heritable diseases.

We show the interference of three different ENM (Carbon black (CB), nano-sized titanium dioxide (TiO$_2$) and multi-walled carbon nanotubes (MWCNT)) with two common genotoxicity assays: the cytokinesis-block micronucleus cytome assay (MN-assay) as a biomarker of chromosome breakage and/or whole chromosome loss [reviewed in 9] and the alkaline single cell gel electrophoresis assay (comet assay) to detect DNA strand breaks [10]. Both assays rely on a fluorescent microscopy read-out and agglomerates of the ENM used clearly either quench or enhance fluorescence intensity. In conclusion critical concentrations above which ENM agglomerate massively, should not be exceeded. Furthermore, alternative methods are needed that circumvent these problems.

2. Materials and Methods

2.1. Cell culture

The human alveolar epithelial cell line A549 (ATCC, CCL-185) [11] was obtained from American Type Culture Collection and grown in RPMI-1640 cell culture medium (Sigma) supplemented with 10% fetal calf serum (FCS, Lonza), 0.2 mg/ml L-glutamine (Gibco), 50 µg/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco) and 100 µg/ml neomycin (Gibco) in humified air at 37°C and 5% CO$_2$.

2.2. Preparation and characterization of nanomaterials

Nano-sized TiO$_2$ and CB suspensions were prepared according to the standard operation procedure “Preparing suspensions of nanoscale metal oxides for biological testing” published at the NanoCare homepage www.nanopartikel.info. Their physico-chemical characterization was done as part of the NanoCare project. Main characteristics are summarized in table 1. MWCNTs (Baytubes®, Bayer Technologies Service) were suspended in 160 ppm Pluronic F127® as described before [12]. Material properties are also detailed in ref. 12.

| material          | provider                        | primary particle size [nm] | size distribution [nm] | BET surface [m$^2$/g] |
|-------------------|---------------------------------|---------------------------|------------------------|-----------------------|
| TiO$_2$           | Evonik Degussa GmbH             | 27                        | 20 – 50000             | 52                    |
| Carbon black      | Evonik Degussa GmbH             | 16                        | 7 – 43                 | 339 +/- 6             |
2.3. Cytokinesis-block micronucleus cytome assay
A549 cells were seeded in a 4-well chambered coverglass slides (Nunc) at a density of 100000 cells per well and cultivated overnight. All samples were treated with 4 µg/ml cytochalasin B (Sigma) for 24 h to block cytokinesis. The resulting binuclearity marks cells that underwent mitosis during the time of treatment. During the 24 h cytokinesis block period cells were treated with 11 µg/cm² MWCNT or 25 µg/cm² CB to analyze nanoparticle interference and genotoxicity. Treatment of cells with 150 µM Methylmethanesulfonate (MMS; Sigma) for 24 h served as a control substance known to induce chromosomal aberrations. Each sample was run in duplicates. After incubation for 24 h at 37°C and 5% CO₂, medium was removed and cells were fixed with 1 ml pre-cooled (-20°C) ethanol for at least 12 h. Samples can be stored in ethanol at -20°C until further processing. To stain the fixed cells, ethanol was removed and cells were air dried for several minutes. Acridine orange (Sigma) was diluted to 0.2 mM in freshly prepared Sörensen’s buffer (24.6 ml 0.06 M Na₂HPO₄·2H₂O and 25.4 ml 0.06 M KH₂PO₄, pH 6.8) and applied for 10 min on a shaking platform in the dark. Cells were washed three times for 5 min with Sörensen’s buffer. Pictures were taken using an Imager M1 epifluorescence microscope (Zeiss).

2.4. Alkaline single cell gel electrophoresis (comet assay)
The comet assay was performed as described before [13]. In brief, A549 cells were seeded in 6-well plates at a density of 25000 cells per well one day prior to treatment. Cells were exposed to 25 µg/cm² TiO₂ or CB for 24 h. Treatment with 15 mM ethylmethanesulfonate (EMS) for 30 min served as a positive control. After incubation, medium was removed, cells were washed with PBS, detached with 0.2 ml Accutase (PAA) and resuspended in 0.3 ml medium. 40 µl of the resulting 0.5 ml cell suspension were mixed with 160 µl 0.5% (w/v) low-melting point agarose in PBS (37°C). 180 µl of this mixture were applied to microscope slides precoated with 1.5% (w/v) agarose in PBS, covered with a coverslip and agarose was allowed to solidify for at least 5 min at 4°C. After removing the coverslips cells were lysed in lysis solution (basic buffer: 2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 1% Na-Sarcosinat, pH 10; freshly added before use: 10% (v/v) DMSO, 1% (v/v) Triton-X-100) for 1 h at 4°C. Slides were placed into a horizontal electrophoresis tray containing alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 20 min without current to allow DNA unwinding. Electrophoresis was performed for additional 20 min at 24 V and 300 mA. Afterwards slides were neutralized for 5 min in TRIS buffer (0.4 M TRIS, pH 7.5), rinsed in H₂O, dehydrated for 5 min in 99% ethanol and dried over night. DNA was stained with 20 µg/ml ethidiumbromide and comets were analyzed using a Nikon Eclipse TS 100 microscope (Nikon) equipped with a Intenslight C-HGFI lamp (Nikon) and a Stingray F046B IRF Jenofilt camera (Allied Vision Technologies). Quantification of head and tail regions was done using the “Comet Assay IV” software (Perceptive Instruments).

3. Results and Discussion
Genotoxicity: two assays and their limitations.

Two different genotoxicity assays were assessed for their nanomaterial compatibility: the cytokinesis-block micronucleus cytome assay (MN assay) and the alkaline single cell gel electrophoresis assay (comet assay). We chose the human alveolar lung epithelial cell line A549 and three different nanomaterials, MWCNT, CB and nano-sized TiO₂, to show typical interference problems with MN-assay as well as comet assay read-outs.

3.1. Cytokinesis-block micronucleus cytome assay.
The MN-assay is a comprehensive system to measure DNA damage, cytostasis and cytotoxicity [9]. Cytochalasin-B treatment allows DNA replication and nuclear division without cytokinesis. This results in a considerable amount of binucleated cells after 24 hours of treatment. After fixation, staining with acridine orange reveals a red fluorescent cytoplasm and green fluorescent DNA in the nuclei. By visual scoring at the fluorescence microscope DNA damage is quantified as the number of
binucleated cells containing an additional micronucleus (arrowhead in figure 1A’). This separate micronucleus results from the breakage of chromosomes or the loss of whole chromosomes due to structural changes in the centromeric region. The proper distribution of these malformed chromosomes during mitosis is thus impossible. The remaining chromosome fragments are enveloped by their own nuclear membrane after completion of mitosis and appear as a small additional nucleus, the “micronucleus”. Increasing numbers of cells with micronuclei could be correlated with an elevated risk to develop cancer [14]. The number of mono-, bi- and polynucleated cells and cells found in mitosis gives a measure of cytostasis. The faster a cell population prolife rates the more bi- and polynucleated cells are expected. Whereas in growth inhibited cultures the number of mono-nucleated cells would be increased. To analyze cytotoxicity the number of dead cells (necrotic and apoptotic) is determined.

Figure 1 shows typical examples of MMS (A, A’) MWCNT (B, B’) and CB (C, C’) treated cells. The black agglomerates that stick firmly to the cells impede the reliable scoring of any of the parameters mentioned above. In particular, tiny micronuclei, the essential genotoxicity characteristic, might be hidden under such agglomerates, falsifying the results drastically. Furthermore, the easily visible agglomerates render it impossible to blind the studies as the scorer recognizes the treated samples at first glance. In conclusion, the MN assay should not be used for black, or otherwise colored, ENM that agglomerate to bigger entities and therefore overlay the fluorescent signal.

Figure 1. MWCNT and CB interfere with MN assay read out. A549 cells were treated with 4 µg/ml Cytochalasin B and 150 µM MMS (A), 11 µg/cm² MWCNT (B) or 25 µg/cm² CB (C) for 24h, respectively. After ethanol fixation cells were stained with acridine orange to visualize nuclei (green) and cytoplasm (red). Representative fluorescence as well as DIC images are shown. Boxes indicate enlarged sections shown in A’ to C’. Arrowhead (A): micronucleus.

3.2. Alkaline single cell gel electrophoresis.
DNA strand breaks as well as alkali-labile sites (abasic sites, resulting from base loss or base excision) are detected in the comet assay. It is based on the electrophoretic separation of DNA fragments
according to their size. Smaller fragments migrate faster and thus out of the former nucleus. Staining with an intercalating fluorescent dye (ethidium bromide) and subsequent microscopic analysis reveal the typical picture of a “comet” with intact DNA remaining in a bright spot, resembling the former nucleus and smaller DNA fragments representing the tail (figure 2B: EMS treated samples show a considerable amount of damaged DNA in the tail region). The fluorescence intensities of head and tail regions of the comets are measured using semi-automated software (Comet Assay IV, Perspective Instruments). Thus, compared to appropriate control samples (figure 2A: untreated sample; figure 2B: positive control EMS treatment), the relative amount of DNA breakage can be quantified.

Figure 2C shows A549 cells treated for 24 h with 25 µg/cm² nano-sized TiO₂. Arrowheads indicate bright spots of nanoparticle agglomerates associated with the DNA. The increase in fluorescence intensity which may result from the photocatalytic properties of these particles disturbs the quantitative image analysis. As a consequence comets that contain such particle spots cannot be included in the analysis. Treatment of A549 cells for 24 h with 25 µg/cm² CB (figure 2D) also reveals interference problems. In this case black spots of CB agglomerates reduce/quench the fluorescence signal equally falsifying the quantitative read out.

Omitting nanoparticle containing comets from the analysis poses major interpretation problems. It is unknown whether particles are able to enter the nucleus already in the intact cell, potentially leading to DNA damage there, or are later on (during the assay procedure) deposited on the naked DNA. Considering the first scenario, omitting comets that contain particle agglomerates would probably underestimate their possible genotoxic effect. This is not true if particles contact DNA after cell lysis. Furthermore, as described for the MN assay, a blinded study is not feasible due to the clearly visible deposits.

Figure 2. Carbon black and TiO₂ nanomaterial deposits interfere with quantitative Comet assay read-out. A549 cells were incubated with 25 µg/cm² TiO₂ (C) or CB (D) for 24h, treated with 15 mM EMS (B) for 30 min or left untreated (A). After lysis and gel electrophoresis DNA was stained with ethidium bromide. Representative fluorescence images are shown. Head and tail regions of a typical EMS induced comet are indicated in (B). Arrowheads in (C) and (D) point to TiO₂ and CB agglomerates, respectively.

Taken together the presence of insoluble nanomaterial agglomerates makes scoring in both assays much more difficult than with soluble chemicals. Two major problems arise from such interference: Firstly, a blinded study is almost impossible, as deposits are easily visible. Secondly, to omit cells with associated or internalized particles might result in missing the cells with the highest DNA damage and therefore lead to false negative results.
4. Conclusions

As a consequence to perform the MN and comet assay with ENM the following precautions should be made:

1) Avoid high, cytotoxic concentrations of ENM. This not only prevents the formation of large visible agglomerates but also prevents false results due to apoptotic DNA fragmentation. The typical apoptotic nuclear fragmentation leads to DNA degradation and therefore false positive results in the comet assay. In contrast, an increase in apoptosis would lead to a false negative result in the MN assay due to the disappearance of micronucleated cells [15].

2) Only cells without nanomaterial deposits should be scored. However, consider the possibility that cells with highest DNA damage might be missed.

3) If possible, results from comet and MN assay should be compared to and completed with methods that are not prone to particle interference – if those exist at all.

In any case the robust and reliable evaluation of genotoxicity caused by nanomaterials is very difficult as soon as agglomerated nanomaterials are visible. In conclusion, the development of robust alternative methods that avoid interference with nanomaterials are desirable and needed.

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