In vitro genotoxicity testing of four reference metal nanomaterials, titanium dioxide, zinc oxide, cerium oxide and silver: Towards reliable hazard assessment

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Abstract

There is serious concern about the potential harmful effects of certain nanomaterials, on account of their ability to penetrate cell membranes and the increased reactivity that results from their increased surface area compared with bulk chemicals. To assess the safety of nanomaterials reliable tests are needed.

We have investigated the possible genotoxicity of four representative nanomaterials, derived from titanium dioxide, zinc oxide, cerium oxide and silver, in two human cell lines, A549 alveolar epithelial cells, and lymphoblastoid TK6 cells. A high throughput version of the comet assay was used to measure DNA strand beaks (SBs) as well as oxidised purines (converted to breaks with the enzyme formamidopyrimidine DNA glycosylase). In parallel, cytotoxicity was measured with the alamarBlue® assay, and the ability of nanomaterial-treated cells to survive was assessed by their colony forming efficiency.

TiO_2 and CeO_2 nanomaterials were only slightly cytotoxic by the alamarBlue® test, and had no long-term effect on colony forming efficiency. However, both induced DNA damage at non-cytotoxic concentrations; the damage decreased from 3 h to 24 h exposure, except in the case of CeO_2-treated A549 cells. ZnO and Ag nanomaterials affected cell survival, and induced high levels of DNA damage at cytotoxic concentrations. At lower concentrations, there was significant damage, which tended to persist over 24 h. The implication is that all four reference metal nanomaterials tested – whether cytotoxic or not – are genotoxic.

A full assessment of nanomaterial toxicity should include tests on different cell types, different times of incubation, and a wide range of (especially non-cytotoxic) concentrations; a test for cell viability should be performed in parallel. Inclusion of Fpg in the comet assay allows detection of indirect genotoxic effects via oxidative stress.

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Introduction

While nanomaterials (NMs) have many and varied beneficial uses, they also have the potential to cause adverse effects on human and animal health and on the environment. The cellular toxicity of NMs can differ markedly from that of the conventional chemicals from which they are derived, depending on factors such as size, shape, stability, mode of synthesis and surface chemistry (1-4). Emerging data suggest that cytotoxicity is caused by induction of oxidative stress (5), hyperinflammation due to over-activation of pro-inflammatory genes, disintegration of cellular components (6), and activation of cell death pathways (7).

Genotoxic effects are regarded as a particularly important aspect of NM toxicity, as DNA damage can lead to mutation and potentially to the development of cancer and birth defects. DNA damage can occur by direct interaction of NPs with the DNA, or as an indirect effect of the induction of oxidative stress. It can be a consequence of cytotoxicity, and so it is important to assess genotoxicity at concentrations of NMs that are non-cytotoxic (8,9).

In view of the large number of NMs currently in use, there is an urgent need to clarify their toxic effects and to elucidate the mechanisms involved in their toxicity. High throughput screening methods aimed at accurately predicting and assessing toxicity are needed. The adoption of high throughput methods for NM toxicity testing allows the assessment of numerous materials at different concentrations and on different types of cells, reduces the effect of inter-experimental variation, makes substantial savings in time and cost, and generates large and valuable data sets - a general strategy recently reviewed by Collins et al. (10).

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We present here the results of a comparative study of four NMs, derived from four different metals: titanium dioxide, zinc oxide, cerium oxide and silver, all used as reference NMs. The NMs were thoroughly characterised in the stock dispersion as well as in appropriate culture media just prior to and after the experiments. Two different human cell types were employed: A549, an alveolar basal epithelial cell line, and TK6 lymphoblastoid cells. After incubation with NMs for 3 or 24 h, cytotoxicity was assessed with the alamarBlue® assay (a redox-based indicator of cellular metabolic activity), and viability was checked by the ability of cells to form colonies when plated out.

Genotoxicity was assessed with the comet assay, which detects both DNA SBs (in its simplest form) and DNA base oxidation (by inclusion of the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (Fpg) which converts oxidised purines to breaks). As a method for measuring DNA damage, the comet assay is deservedly popular on grounds of sensitivity, accuracy, simplicity and economy; but it is limited in the number of samples that can be analysed in one experiment by the size of the electrophoresis tank, typically accommodating 20 slides with one or two gels each. We have applied a high throughput version with 12 mini-gels per slide (Figure 1), greatly increasing the capacity; indeed, only by using this approach could our study of four NMs, two cell types, several doses, and two time points (with a consistent set of experimental protocols) be managed. The 12 mini-gels were already used in our previous studies with iron oxide NMs (11), silver NMs of different sizes (3), and six nanosilver NMs of three different charges, each with different surface properties (4,12).

This investigation was an integral part of the Europe-wide FP7 project NANoREG (supported by a Norwegian Research Council project grant NorNANoREG), which aims to provide a regulatory framework for assessment of all aspects of potential NM toxicity.

Materials and Methods

Chemicals

Most reagents for cell culture were purchased from Sigma-Aldrich; culture media, fetal bovine serum (FBS), heat inactivated horse serum (HS), antibiotics and other chemicals used for cell cultivation. SybrGold DNA stain was purchased from Invitrogen (Life Technologies™, USA).

Nanomaterials

Nanosilver, Ag NM300K (spherical <20nm, solid content 10.16% by weight; particle size: 15 nm; D90 <20nm (i.e. 90% <20nm)) was obtained from Fraunhofer-Gesellschaft (Institute for Molecular Biology and Applied Ecology, Aachen, Germany). It was purchased dispersed in Tween20/PEG. The solution NM300disp used to disperse Ag NPs was also purchased from Fraunhofer and served as control material for Ag NM300K.

Titanium dioxide, TiO_2 NM100, zinc oxide, ZnO NM110 and cerium oxide, CeO_2 NM212 were obtained from the Joint Research Centre (Ispra, Italy). Following the Nanogenotox dispersion protocol (www. nanogenotox.eu/files/PDF/ web %20nanogenotox%20 dispersion %20protocol.pdf.) each NM (except Ag NM300k which was purchased in liquid) was pre-wetted in 0.5% ethanol and dispersed in 0.05% bovine serum albumin (BSA) in MilliQ water to obtain a stock suspension of 2.56 mg/ml. The suspension was then sonicated using two sonicator models; BransonS-450D and Labsonic P (Sartorius Stedim Biotech, Göttingen, Germany) for 15 min (100 % cycle, 100 W) on ice. Both were calibrated previously to achieve the same acoustic power of 7.35±0.05 W, as required for the NANoREG project. The resulting stock suspension from the dispersion procedure was serially diluted to achieve a logarithmic range of concentrations before adding it to the cells already seeded in 96- or 6-

well plates. Final concentrations were from 0.01 to 75 µg/cm^2 for adherent A549 cells, equivalent to 0.14 to 140 µg/ml for TK6 cells in suspension culture.

These NMs were thoroughly characterized in the course of the Nanogenotox EU project (2012) and further characterised within NANoREG (Table 1). Size distributions were confirmed by Dynamic Light scattering (DLS) using a Zetasizer Nano ZS and Nanoparticle Tracking Analysis (NTA) using Nanosight NS500 (both from Malvern Instruments) (Table 2).

Table 1. Characterisation of nanomaterials

Material code	**Core material**	**Polymorph**	**Product type**	**Morphology**	**XRD size [nm]**	**TEM** **Diameter (nm)**
1.NM100	TiO_2	anatase	Powder	-	56.7 to > 100	110±57
2.NM110	ZnO	zincite	Powder	-	>100	147±149
3.NM212	CeO_2	cerite	Powder	-	NA	33
4.NM300K	Ag	metallic	Dispersion	sphere	14±2	16.7±4.0

The listed analytical data were mainly generated by the EAHC NANOGENOTOX project, the EU FP7 project ENPRA, or retrieved from JRC data-reports on OECD WPMNM

Table 2. Particle characteristics in terms of particle size in stock and culture medium, assessed by NTA and DLS

		TiO_2 NM100	CeO_2 NM212	ZnO NM110	Ag NM300K
NTA	Particle size distribution in:				
	Stock	195 ± 30	218 ± 68	189 ± 62	65 ± 14
	DMEM 0 h	196 ± 37	174 ± 32	148 ± 25	65 ± 11
	DMEM 3 h	189 ± 47	214 ± 29	231 ± 14	66 ± 13
	DMEM 24 h	170 ± 50	86 ± 15	193 ± 70	66 ± 7
	RPMI 0 h	179 ± 61	191 ± 1	243 ± 8	75 ± 1
	RPMI 3 h	194 ± 39	205 ± 3	252 ± 2	77 ± 5
	RPMI 24 h	157 ± 68	194 ± 0	251 ± 4	112 ± 10
DLS	Mean average in stock	219 ± 1.91	226.7 ± 11,14	210 ± 3.25	70.54 ± 0.84
	PDI	0.17 ± 0.015	0.28 ± 0.04	0.14 ± 0.05	0.26 ± 0.01

The results are mean±SD of three independent experiments.

Cell culture and treatment

The human alveolar basal epithelial cell line A549 was obtained from the European Collection of Cell Cultures (ECACC). The cells were cultured in 75 cm^2 flasks in low glucose DMEM with 9% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 4 mM L-glutamine in a humidified atmosphere of 5% CO_2 at 37°C. The cells were routinely sub-cultured every second day or when they reached about 70-80% confluence.

The human lymphoblastoid cell line TK6, purchased from the ECACC, was cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS, 100 U/ml penicillin and

100 µg/ml streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C. The cells (grown in suspension) were routinely diluted to 2 x10^5 cells per ml.

Cells were treated with different concentrations of the selected NMs for 3 or 24 h (alamarBlue® and comet assays) and chronic exposure for 9-12 days (CFE). The concentration range to determine cytotoxicity and genotoxicity was between 0.01–75 μ g/cm^2 for adherent cells A549 (considering surface area of 96-well plate format) and for cells in suspension, TK6, the range of concentrations was between 0.14 and 140 µg/ml.

The dispersant material NM300Kdisp, corresponding to the vehicle of Ag NM300, including all components except silver, was tested at the concentration corresponding to the highest concentration of nanosilver as an additional control. The negative control was culture medium. The vehicle, 0.05 % BSA in water, was also tested. As positive controls for alamarBlue®, hydrogen peroxide (1mM, 3 h exposure) and staurosporine STS (5nM, 24h exposure) were used.

Cytotoxicity assay using alamarBlue®

AlamarBlue® measures cytotoxicity through a colorimetric response to the intracellular reducing metabolism of living cells. The conversion of resazurin (oxidised form) to resorufin (reduced form) results in colorimetric and fluorescence changes; resazurin is blue and non-fluorescent whereas resorufin is red and highly fluorescent.

A549 cells were washed, trypsinised, counted and seeded in a 96-well plate (1 × 10^4 cells /well), and incubated at 37 °C. After 24 h, cells were exposed for 3 h or 24 h to NMs at concentrations between 0.01 and 75 µg/cm^2 and to the positive controls. The treatment was completed by removing the medium and washing the cells twice with PBS. Cells were then incubated for 3 h with fresh culture medium supplemented with 10 % alamarBlue®, after

which 100 µl of medium from each well was transferred to a 96-well black polystyrene microplate (duplicate or triplicate aliquots). Fluorescence (excitation 530 nm, emission 590 nm) was measured on a FLUOstar OPTIMA microplate reader.

Clonogenic assay for cytotoxicity (colony forming efficiency)

The ultimate index of cytotoxicity is loss of cell viability, and this can be measured by the ability to survive and form colonies (colony forming efficiency, CFE). CFE is a quantitative label-free method and therefore is suitable for cytotoxicity testing of NMs without interference with the readout – a common problem with colorimetric assays. The CFE assay was optimized and standardized for NM testing, validated in an inter-laboratory comparison and shown to be sufficiently sensitive to detect potential NM toxicity (13)(14). The assay was modified for higher throughput by reducing the number of cells to 50 per well and applying a 6-well plate format. The test allows for detection of cytotoxic effects (reduction in number of colonies) as well as cytostatic effects (reduction in colony area).

A549 cells were washed in PBS, trypsinized, counted, and seeded at 50 cells per well in 6-well plates 1-3 h before exposure. NMs were added to six replicate cultures per concentration (ranging from 0.01 to 75 µg /cm^2) and cells were incubated at 37 °C for 9-12 days, NMs being present throughout. The cells were fixed and stained with 1% methylene blue, washed with water and dried before manual counting of colonies. Two or three independent CFE assays were performed for each NM. The results were normalized to the unexposed control (set to 100 % plating efficiency):

$$CFE\% = \frac{number\ of\ colonies\ in\ exposed\ cultures}{number\ of\ colonies\ in\ unexposed\ cultures}\ x\ 100\ \%$$

Comet assay (high throughput)

Twenty four hours after seeding A549 and TK6 cells in 96-well plates (1×10^4 cells /well), they were exposed to freshly dispersed NMs at a range of concentrations (at least six, between 0.1-75 μg/cm^2 (A549) and 0.14 and 140 μg/ml (TK6) for 3 h or 24 h. Depending on results of cytotoxicity tests, the highest concentrations were in some cases omitted. After exposure, cells were washed with PBS and trypsinized in the case of adherent A549 cells. Using an electronic multichannel pipette, 50 μl ($0.5x10^4$ cells) were transferred to a 96-well U-bottom plate and mixed with three volumes of LMP agarose (0.8 % in PBS) at 37°C. From this mixture 10 μl drops were placed on glass slides (pre-coated with 0.5 % standard melting point agarose) - 2 drops per concentration, 12 drops per slide; see Figure 1. After 5 min at 4°C slides were immersed in cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 10 % Triton X-100, pH 10) overnight. After lysis, slides were placed in alkaline solution (0.3 M NaOH, 1 mM EDTA) for 20 min, followed by electrophoresis at 1.25 V/cm for 20 min in a standard horizontal electrophoresis tank. Slides were then washed in PBS followed by water and allowed to dry overnight. Slides were stained with SybrGold® (Invitrogen) diluted at 1 μl/ml in TE buffer (10 mM TrisHCl, 1 mM Na_2EDTA, pH 7.5–8), covered with a cover slip and examined under a fluorescence microscope (Leica DMI 6000 B). Images of comets were scored using Comet Assay IV software (Perceptive Instruments), calculating median % DNA in tail from 50 comets per gel as a measure of DNA SBs.

For DNA base oxidation detection we used the comet assay modified by inclusion of a post-lysis incubation with Fpg (prepared as a crude extract from *Escherichia coli* with an over-producing plasmid). Fpg converts oxidised purines to SBs. After lysis, slides were washed with Fpg buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0) without enzyme, placed in a humid box, and 30 μl of Fpg previously diluted in this buffer was added to each gel. Slides were covered with polypropylene film and incubated for 30 min at 37 °C. After enzyme treatment, the slides were incubated in alkali and

electrophoresed as described above. Net Fpg-sensitive sites were calculated as the difference in % DNA in tail between samples with Fpg incubation and samples without incubation.

All experiments included positive and negative control (standard reference) cells. As a positive control for SBs, cells were treated with H_2O_2 at 100 μM, for 5 min on ice. As positive and negative controls for the Fpg assay, we used aliquots stored frozen at -80°C, from a single batch of cells, either untreated, or with a known amount of 8-oxoguanine induced by incubating cells with photosensitizer Ro19-8022 (Hoffmann La Roche) at 2 μM in PBS, and irradiating with visible light (500 W halogen source, 30 cm from cells) for 5 min on ice.

Statistical analysis

One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows, Data are expressed as average of 2-3 independent experiments ± SD for alamarBlue® and comet assay results and ± SEM for CFE assay.

Results

Nanomaterial characterization

In addition to the characterization of the NMs carried out in the course of the EU Nanogenotox project (see Materials and Methods), we carried out our own characterization within 30 min after dispersion of the NMs to determine the actual average size (NTA, DLS). Size distribution of these NMs in different culture media was also evaluated using NTA-NS500, and found to be stable over times 0, 3 and 24 h (Table 2, Figure 2). Results with NTA and DLS were consistent.

Cytotoxicity of NMs measured by the alamarBlue®assay

Cytotoxicity assessments using the alamarBlue® assay are shown in Figure 3. Exposure of A549 cells for 3 or 24 h to TiO_2 NM100, or CeO_2 NM212 did not decrease cell viability (expressed as % cell functionality) significantly, although TiO_2 NM100 produced a slight decrease in viability at 3 µg/cm^2 which after 24 h was reversed. However, ZnO NM110 and Ag NM300K were very cytotoxic at high concentration and induced significant decreases in viability at moderate concentrations; 10 µg/cm^2 and above for zinc and 3 µg/cm^2 and above for silver after both exposure times. The positive controls (hydrogen peroxide (1mM) for 3 h exposure and staurosporine STS (5nM) for 24h exposure) both induced very high cytotoxicity, with 25% cell functionality for H_2O_2 and 45 % cell functionality for Staurosporine (data not shown). No decrease in cell viability compared with control cells was detected in cells treated with the dispersion solution for Ag NM300K (data not shown).

Cytotoxicity of NMs measured as colony forming efficiency

CFE is expressed as the ratio of the number of colonies in exposed cultures relative to unexposed. Colonies were counted after 9-12 days of continuous exposure. A compound is considered cytotoxic when the % CFE is below 80%. The negative control was DMEM culture medium. The vehicle, 0.05 % BSA in water, was also tested and found to be comparable to the negative control (not shown). Of the four different NMs tested, TiO_2 NM100 and CeO_2 NM212 had no effect on CFE. However, both ZnO NM110 and Ag NM300K were cytotoxic, in a concentration-dependent manner (Figure 4). After treatment with these two NMs the colonies were very small, indicating a cytostatic effect. Ag dispersion solution was tested and found to be cytotoxic at the highest concentrations; therefore the cytotoxicity of silver at the highest concentrations could be influenced also by the dispersion

solution (data not shown). The number of colonies formed was reduced by ZnO NM110 at 0.3 µg/cm^2 or above and by Ag NM300K at 0.1 µg/cm^2 and above.

NM-induced DNA damage measured by the modified comet assay

The comet assay measures DNA damage at the cellular level. While the basic comet assay detects SBs, a common modification – incorporating digestion with a lesion-specific endonuclease after the lysis step – allows detection of damaged bases. Fpg is used to measure the effect of oxidative stress on DNA as its primary substrate is the oxidised base 8-oxoGua.

We employed a high throughput version of the comet assay, with twelve mini-gels placed on a standard microscope slide, in order to be able to test a large number of NM concentrations of up to four types of NM in a single experiment - thus reducing the chance of experimental variation affecting results. The four NMs were tested at at least five concentrations for DNA SBs and Fpg sites induced in TK6 and A549 cells over periods of 3 h and 24 h of exposure. Results for the four NMs are shown in Figures 5 and 6.

TiO_2 NM100 are relatively non-toxic NPs, with no long-term effect on CFE. In both cell types, concentration-dependent DNA damage - both SBs and Fpg-sites - was detected at 3 h (though the concentration dependence was rather irregular in the case of Fpg-sites). A549 cells showed more damage than did TK6 cells, but in both cases there was a substantial decrease in total damage (SBs plus Fpg sites) at 24 h.

The other NM with limited cytotoxicity is CeO_2 NM212. Again, a concentration-dependent increase in DNA damage was seen in both cell types after 3 h, and again the rise in Fpg sites was not maintained over the whole range of concentrations. There was a contrast in the effects of these NMs in the two cell types at 24 h; while there was a decrease in DNA damage in TK6 cells, damage (both SBs and Fpg-sites) increased almost two-fold in A549 cells.

ZnO NM110 induced high levels of damage at cytotoxic concentrations; in the case of TK6 cells it reached the level of saturation of the comet assay at 14 µg/ml (almost 100% DNA in tail after lysis alone) which means that it is not possible to estimate by subtraction the amount of base oxidation (i.e. Fpg sites). This high level of damage - presumably secondary to cytotoxicity - was maintained at 24 h, while at lower concentrations, damage of both sorts was reduced to near background levels. By contrast, in A549 cells there was less damage owing to cytotoxicity; a clear concentration-dependent increase was observed after 3 h and 24 h, with a decrease in Fpg sites at 24 h, but no decrease in SBs.

Ag NM300K also shows high (saturating) damage at cytotoxic concentrations in TK6 cells. At lower concentrations, there was a weak concentration-dependence, and little change at 24 h. A549 cells showed similar levels of SBs at 3 h and 24 h, while Fpg sites increased. No significant increase in DNA damage (either SBs or Fpg sites) was observed in cells treated with the dispersant for silver (data not shown).

In Table 3, differences in the responses of the two cell types to the four tested NMs in the two cell types are illustrated by focusing on the total DNA damage (% tail DNA in presence of Fpg, without subtracting SBs) at a common concentration of 1 µg/cm^2 /1.4 µg/ml after 3 and 24 h. Overall, A549 cells appear more sensitive to DNA damaging effects. TiO_2 NM100-induced damage decreased by 24 h in both cell types, as is the case for ZnO NM110. Damage resulting from exposure to Ag NM300K remained more or less the same over time in both cell types. However, while damage induced by CeO_2 NM212 increased with time in A549 cells, it decreased in TK6 cells.

Table 3. Comparison of responses of the two cell lines, TK6 and A549, in terms of total DNA damage (SBs plus Fpg-sites) at 3 and 24 h of exposure to 1µg/cm^2 (equivalent to 1.4 µg/ml) of each NM.

	SBs+Fpg-sites (% tail DNA)	
3 h exposure	**TK6**	**A549**
TiO_2 **NM100**	23	40
ZnO NM110	37	58
CeO_2 **NM212**	21	15
Ag NM300K	29	37
24 h exposure	**TK6**	**A549**
TiO_2 **NM100**	9.0	17
ZnO NM110	8.0	25
CeO_2 **NM212**	9.1	27
Ag NM300K	25	46

Discussion

There is a mismatch between the speed with which new NMs appear on the market and the current low-throughput, time-consuming and laborious methods for evaluating their toxicity (15). The FP7 project NANoREG (of which the present work is a part) aims to develop testing strategies for NMs that can be built into a hazard assessment and regulatory framework. One task of the project focuses on development of high throughput methods – as reviewed (10). The need for high throughput methods and automation was already highlighted in the FP7 NanoTEST project (3,4,9,12,16,17).

We report here a concerted effort to compare and contrast four distinct NMs, in terms of their genotoxic effects on two different cell types. Few previous studies have compared the genotoxic effects (both DNA SBs and oxidised bases) of a variety of thoroughly characterised NMs, over such a wide range of concentrations (three orders of magnitude), with different periods of exposure, in different cell types.

We selected for the study reference NMs of varied chemical composition, and our aim was to produce benchmark data concerning their potential biological hazards, if any. We followed recommended practice in carrying out a detailed characterisation of each NM, in its dispersion medium, immediately before, during and after applying to the cells (9) - a time-consuming step that cannot be fully adapted to high throughput. The demands of cell culture, and the need to assess cytotoxicity using two approaches (metabolic function tested with alamarBlue®, and viability by CFE), also limit the number of NMs that can realistically be tested at one time. However, we exploited the high throughput comet assay to measure DNA damage. The number of samples that can be analysed in one electrophoresis run was increased 6-fold by employing 12 mini-gels rather than two large gels per slide. This allowed us to test up to six concentrations of all four NMs in one experiment, measuring both DNA SBs and Fpg-sensitive sites.

It is important to relate the capacity of NMs to damage DNA to their cytotoxicity. DNA damage can be secondary to cytotoxicity (for example, through release of nucleases), and so to investigate genotoxicity it is recommended to study concentrations that are non-cytotoxic. However, what is the best test of cytotoxicity? The alamarBlue® assay measures the functioning of a particular metabolic pathway, and is a good guide to short-term cytotoxicity. (Care should, however, be taken when using this test, as it is prone to interference of the NMs at the read-out stage, as reported (18).) All four NMs tested here begin to give indications of DNA damage at concentrations well below cytotoxic concentrations, so we can confidently

say that the genotoxic effects are independent of cytotoxicity. Long-term viability is assessed with the CFE assay, already pre-validated for NMs (13) with consistent results between laboratories. In this assay, two of the NMs (TiO_2 NM100 and CeO_2 NM212) had no effect. Does this mean that these NMs can be given a clean bill of health? Not necessarily, as the fact that DNA damage was induced indicates a potential risk of mutagenesis; even if the damage was completely repaired, repair is not always faithful. Therefore, for a complete picture of genotoxicity, we need to perform assays covering other relevant endpoints, such as gene mutation, structural chromosomal damage and aneuploidy which are indicative of genotoxic carcinogens. Cell transformation assays can give additional valuable information about genotoxic and non-genotoxic carcinogens (19).

There are numerous reports of DNA SBs and/or oxidised bases detected with the comet assay in cells treated with a variety of NMs. TiO_2 is most often the subject of study, and results vary, with some reports of SBs, some of oxidised bases, and some showing both. Variations in conditions (particularly concentrations of NPs and period of incubation) make comparisons with the present study difficult. Damage seen at high concentrations (1 mg/ml) (20) could be secondary to cytotoxicity. However, some papers report damage at concentrations as low as 1 or 4.8 μg/ml (21,22). Inclusion of Fpg in the comet assay gives results that shed light on the mechanism of genotoxicity. We can say, for instance, that in A549 cells treated with Ag NM300K, oxidation of DNA bases continues during 24 h incubation, leading to an accumulation of Fpg sites, whereas in other cases there is a clear decrease in the frequency of Fpg sites.

Comparison of 3 h and 24 h exposures illustrates the differences between NMs and between cell types - and raises questions which cannot be answered at present. In terms of cytotoxicity (measured with the alamarBlue® assay only in A549 cells), cells show improved function

after 24 h compared with 3 h (except in the case of ZnO NM110 which shows no change at moderate concentrations). Thus cells have a capacity to deal with effects of NMs - even though they are still present in the cells, as has previously been shown (3,12). Also in the case of DNA damage, there is generally a decrease at 24 h. Does the decrease represent an induction of DNA repair pathways, or a sequestration of NMs? Dilution of NMs by cell division will have a significant effect over 24 h. But in cases such as ZnO NM110 in TK6 cells, damage decreases to a level close to background - even at concentrations (1.4 and 4.2 μg/ml) that are cytotoxic.

A549, the alveolar basal epithelial cell line, seems to be the most popular human cell line for NM toxicity testing - representing, as it does, a principal target tissue for exposure. Ursini et al. (23) compared the effects of pristine and carboxylated multi-walled carbon nanotubes (MWCNTs) in the two cell lines. At the highest concentration used, 40 μg/ml, only pristine MWCNTs induced SBs in A549 cells, while in BEAS-2B cells, both types of MWCNT induced significant damage. No Fpg-sensitive sites were detected in either cell line with either type of NM. An investigation of TiO_2 NPs by this group (24) showed both SBs and Fpg-sensitive sites in A549 cells at 2 h, 40 μg/ml, but no damage at 24 h – underlining the importance of looking at different times of exposure. In BEAS-2B cells, TiO_2 did not induce significant damage of either kind. Cobalt oxide (Co_3O_4) NPs at 2 h, 40 μg/ml caused SBs in both A549 and BEAS-2B cells, with no sign of a decrease at 24 h; Fpg sites were also induced, at 20 μg/ml in A549 cells and as low as 5 μg/ml in BEAS-2B cells (25).

The importance of variation between cell types was recognised by Cowie et al. (8), who tested six NMs on several cell lines of different origins, representing eight different organs; they included human lymphocytes, TK6 lymphoblastoid cells, COS-1 monkey kidney cells, BeWo b30 choriocarcinoma cells, BEAS-2B bronchial epithelial cells, HCEC endothelial cells

derived from the central nervous system as well as primary hepatocytes. HCEC cells showed a similar response to cells of blood origin or kidney cells in terms of TiO_2 and oleic acid coated iron oxide (OC-Fe_3O_4) NM-induced DNA damage. BeWo b30 cells showed a significant positive response to TiO_2 and OC-Fe_3O_4 NMs though only at one non-cytotoxic concentration. TK6 cells, human lymphocytes, BeWo b30 and kidney cells seemed to be the most reliable for detecting a concentration-response. These results agree with our present findings that the degree of induction of DNA damage *in vitro* is dependent on NM type, time of exposure and concentrations (8).

In conclusion, some general statements can be made, for consideration when planning any investigation of genotoxic effects of NMs:

- A 24 h incubation alone is not sufficient to detect damaging effects of certain NMs on DNA; short and long term exposure should always be performed.
- Responses to NMs can vary depending on the cell type selected for testing.
- Genotoxicity should be assessed at concentrations that are not cytotoxic (setting a threshold of perhaps 80% viability by a test such as alamarBlue®).
- The use of Fpg allows detection of oxidative damage to DNA, which would otherwise be missed.
- A small increase in concentration can have profound effects in terms of genotoxicity.
- The use of a high throughput version of the comet assay to measure DNA damage increases robustness and reduces experimental variation.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure legends

Figure 1. Experimental design to test up to six nanomaterials NMs using one cell line, two times of exposure (3 h and 24 h) and at least three endpoints; alamarBlue® assay (AB), colony forming efficiency assay (CFE) and comet assay (CA).

Figure 2. Size distribution (NTA) in stock dispersion and in culture medium, at 0, 3 and 24 h.

Figure 3. Effect of NM100, NM110, NM212 and NM300K NMs on the viability of A549 cells measured by the alamarBlue®assay (AB). Cells were incubated in the absence and presence of different concentrations of each NM (0.01–75 µg/ml) and the cell viability was determined after 3 h exposure (black bars) and 24 h exposure (grey bars). Untreated cells were used as negative control and cell functionality is expressed relative to negative control (set at 100%). Cells exposed to hydrogen peroxide (1mM) for 3 h and Staurosporine 5 nM for 24 h were used as positive controls, giving 25 and 40% cell functionality, respectively (data not shown). Data are presented as the means of three independent experiments ± SD. *P* values indicate statistically significant differences from the unexposed cells; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ***$p < 0.0001$.

Figure 4. Cytotoxic effects of NM100, NM110, NM212 and NM300K measured by the colony forming efficiency (CFE) assay in A549 cells after continuous NM exposure . Fifty cells were seeded per well on a series of 6-well plates and treated with different concentrations of each NM. Cell survival is expressed relative to negative control (set at 100%). Fifty cells per well were exposed to the NMs at a concentration range from 0.01 – 75 µg/cm^2. Mean CFE is shown +/- SEM from 2-4 independent experiments (six replicas, i.e. one plate per concentration).

Figure 5. DNA damage (mean % DNA in tail) induced in TK6 cells by four NMs, after treatment for 3 h or 24 h: Strand breaks (SBs), SBs plus oxidised purines (SBs+Fpg) and net oxidized bases (Net Fpg). Data are presented as mean values ± SD. *P* values indicate statistically significant differences from unexposed cells; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ***$p < 0.0001$. Positive control for SBs using H_2O_2 (100 µM, 5 min in ice) and for Fpg sites

using Ro19-8022 (2 μM, plus visible light, 5 min, on ice) gave 62% and 55% DNA in tail, respectively (data not shown). x: Level of DNA damage could not be fully evaluated,

Figure 6. DNA damage (mean % DNA in tail) induced in A549 cells by four NMs, after treatment for 3 or 24 h: Strand breaks (SBs), SBs plus oxidised purines (SBs+Fpg) and net oxidized bases (Net Fpg). Data are presented as mean values± SD (except for Ag NM300K, where the range of duplicates is shown). *P* values indicate statistically significant differences from unexposed cells; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ***$p < 0.0001$. Positive control for SBs using H_2O_2 (100 μM, 5 min in ice) and for Fpg sites using Ro19-8022 (2 μM, plus visible light, 5 min, in ice) gave 76% and 69% DNA in tail, respectively (data not shown). x: Level of DNA damage could not be fully evaluated.

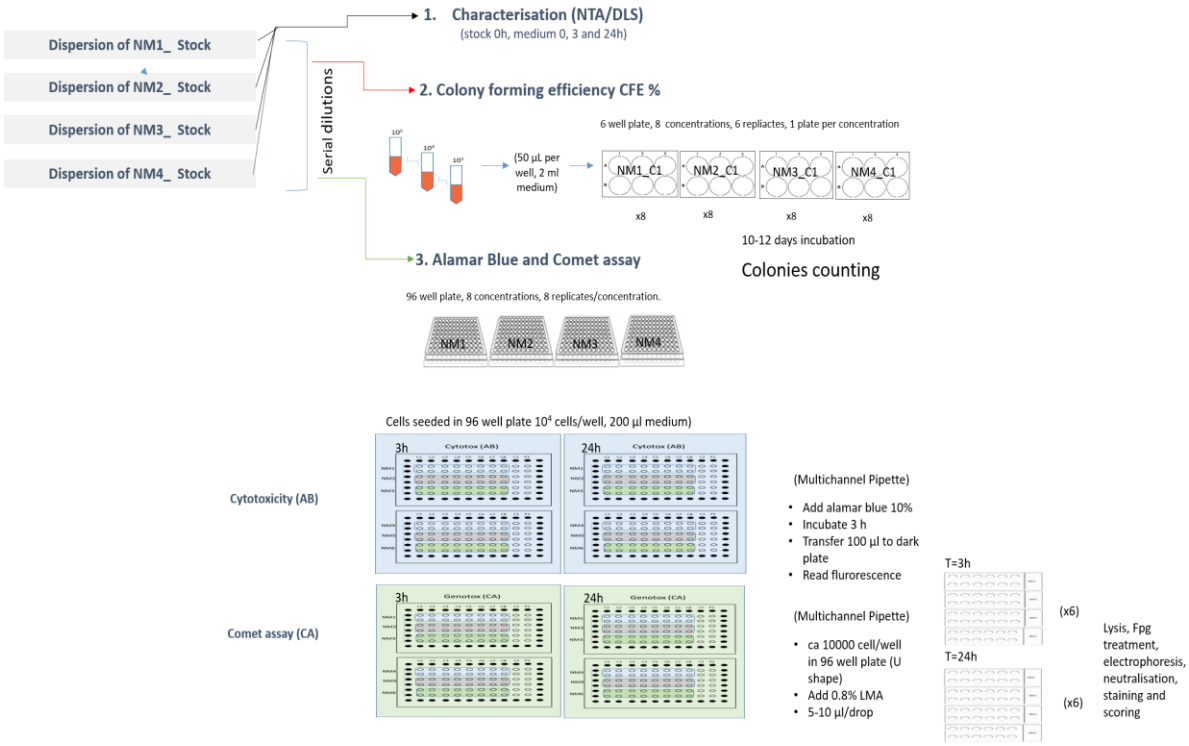

Figure 1.

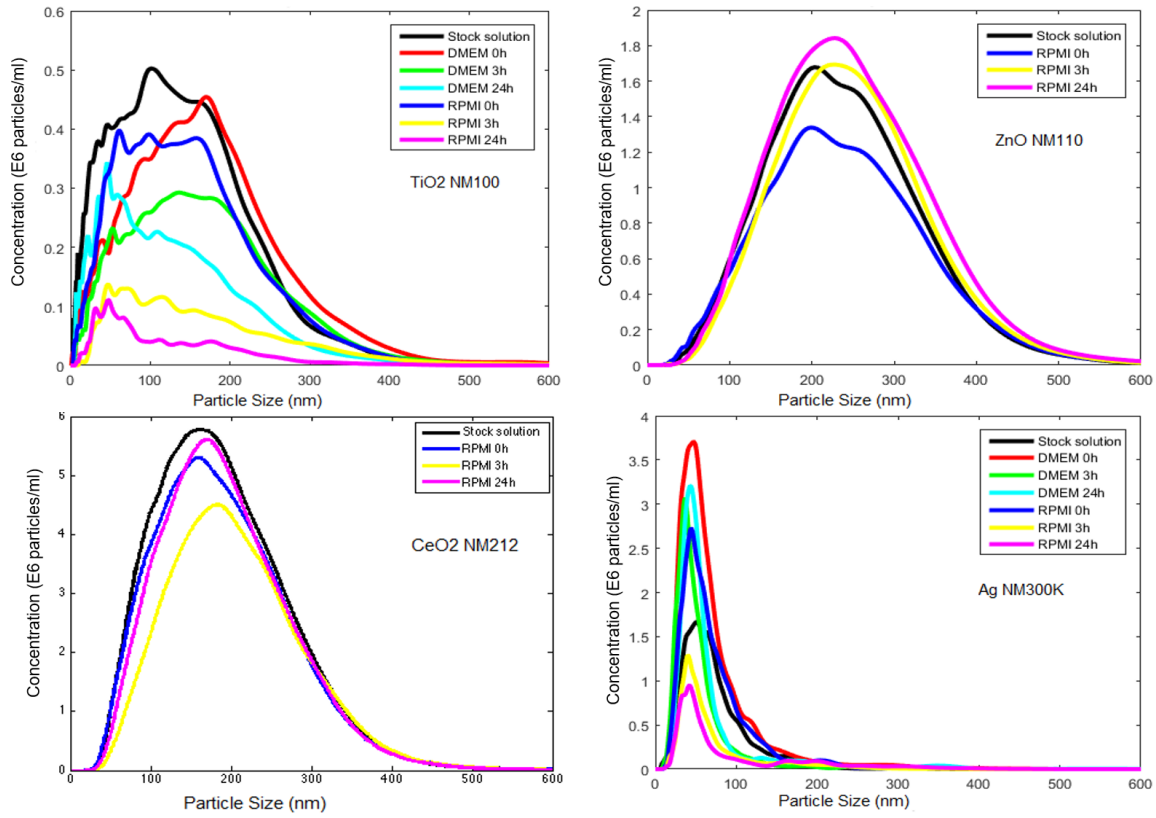

Figure 2.

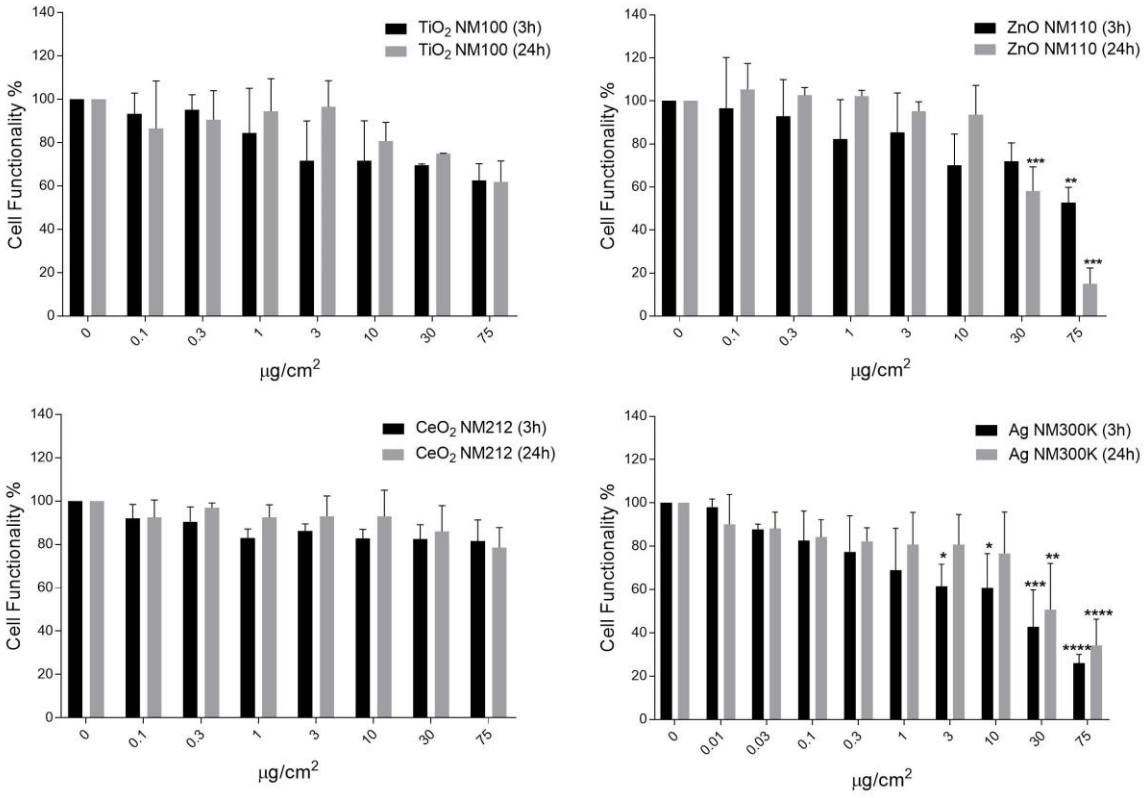

Figure 3.

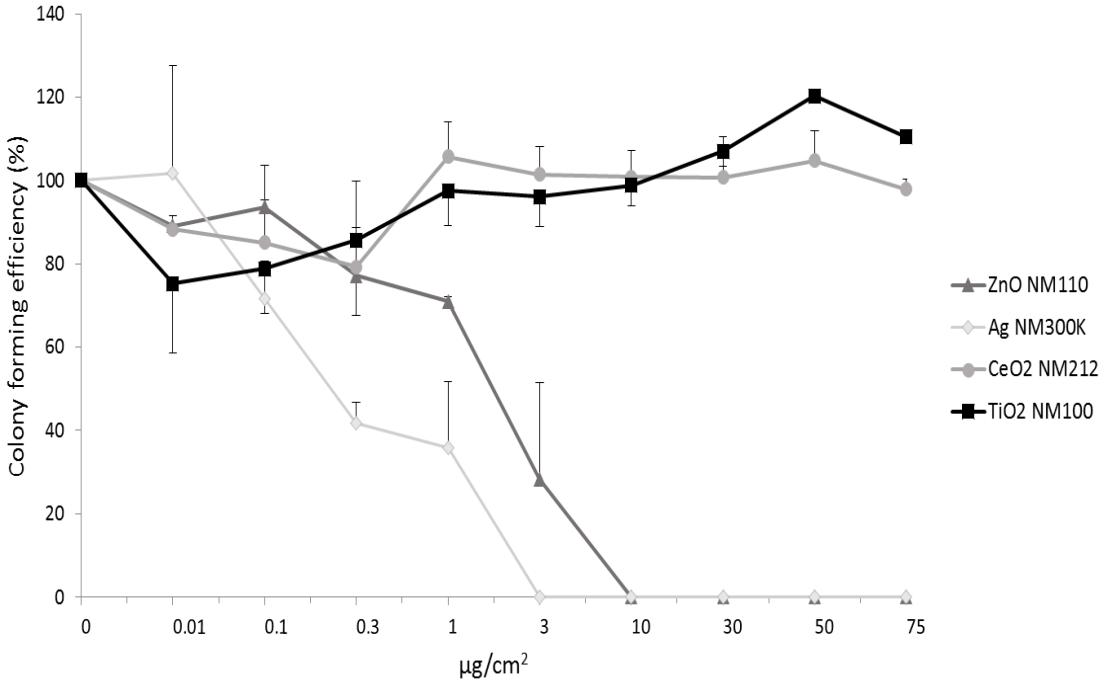

Figure 4.

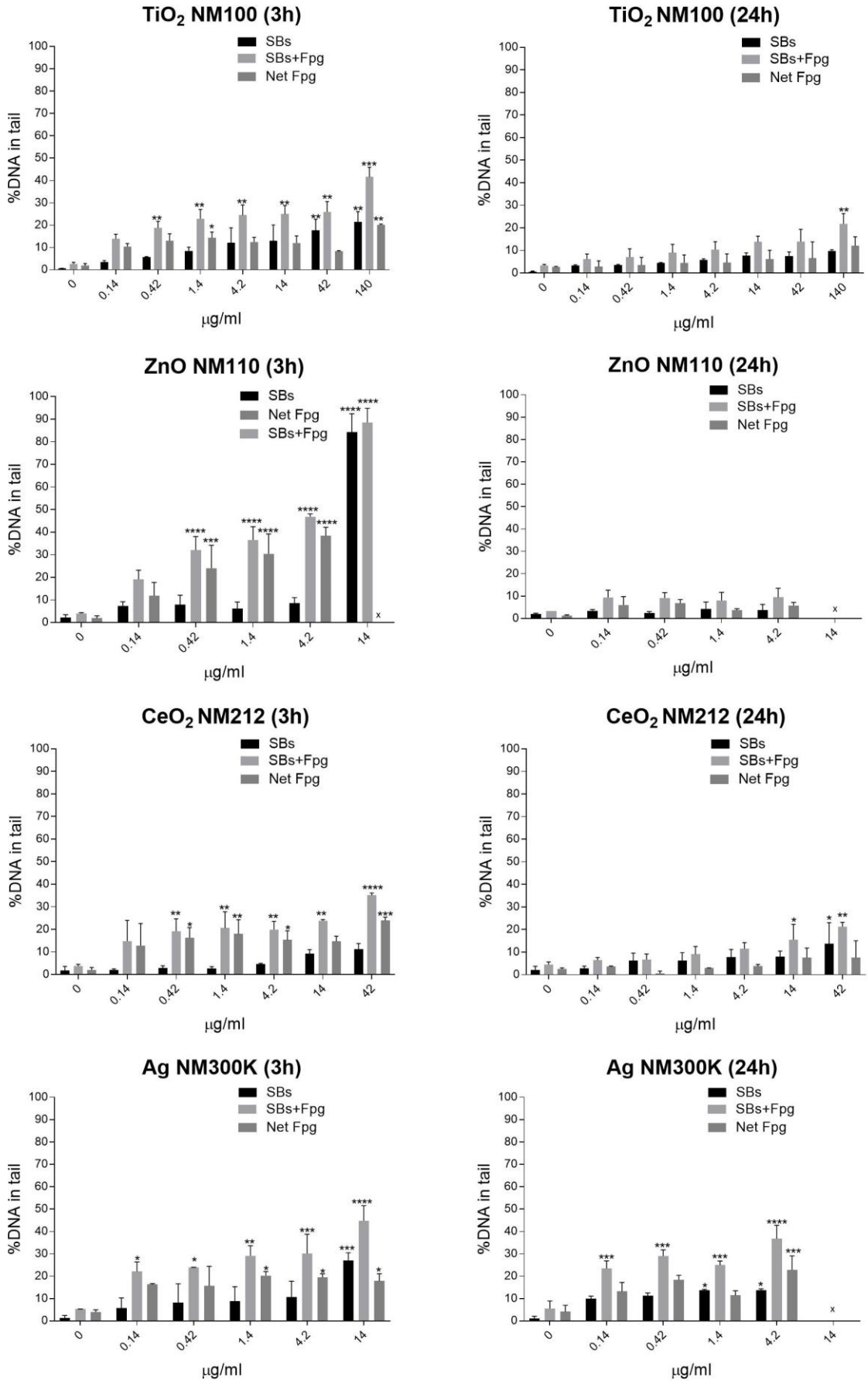

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Figure 5.

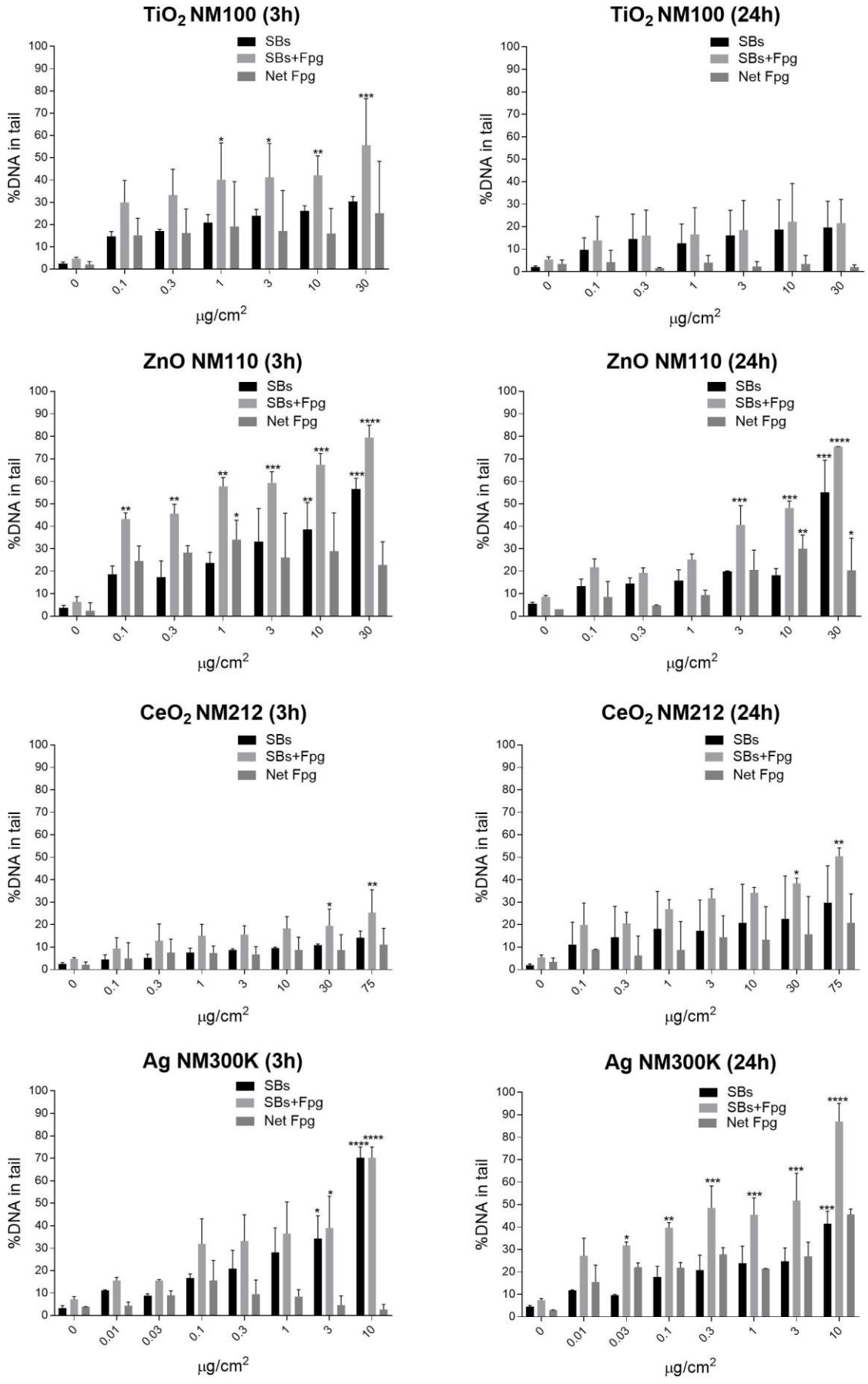

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Figure 6.