Journal of Environmental Monitoring

Cite this: *J. Environ. Monit.*, 2012, **14**, 455

www.rsc.org/jem

PAPER

Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related *in vitro* cytotoxicity and genotoxicity†

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Received 13th September 2011, Accepted 23rd December 2011
DOI: 10.1039/c2em10746e

The published results on nanoparticles cytotoxicity and genotoxicity such as titanium dioxide nanoparticles (TiO_2 NPs) are inconsistent, and often conflicting and insufficient. Since different parameters may have impact on the toxicity results, there is need to lay stress on detailed characterization of NPs and the use of different testing conditions for assessment of NPs toxicity. In order to investigate whether dispersion procedures influence NP cytotoxicity and genotoxicity, we compared two protocols giving TiO_2 NP dispersions with different stability and agglomeration states. Detailed primary and secondary characteristics of both TiO_2 NP dispersions in culture media were carried out before toxicological testing; TK6 human lymphoblast cells, EUE human embryonic epithelial cells and Cos-1 monkey kidney fibroblasts were used to assess cytotoxicity (by trypan blue exclusion, proliferation activity and plating efficiency assays) and genotoxicity (by the comet assay). DNA strand breaks were detected by the alkaline comet assay. DNA oxidation lesions (especially 8-oxo-7,8-dihydroguanine, 8-oxoG) were measured with a modified comet assay including incubation with specific repair enzyme formamidopyrimidine DNA glycosylase (FPG). The TiO_2 NPs dispersion with large agglomerates (3 min sonication and no serum in stock solution) induced DNA damage in all three cell lines, while the TiO_2 NPs dispersed with agglomerates less than 200 nm (foetal serum in stock solution and sonication 15 min) had no effect on genotoxicity. An increased level of DNA oxidation lesions detected in Cos-1 and TK6 cells indicates that the leading mechanism by which TiO_2 NPs trigger genotoxicity is most likely oxidative stress. Our results show that the dispersion method used can influence the results of toxicity studies. Therefore at least two different dispersion procedures should be incorporated into assessment of cyto- and genotoxic effects of NPs. It is important, when assessing the hazard associated with NPs, to establish standard testing procedures and thorough strategies to consider the diverse conditions relevant to possible exposures.

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† Presented at AIRMON 2011, the 7th International Symposium on Modern Principles for Air Monitoring and Biomonitoring, June 19–23, 2011, Loen, Norway.

Environmental impact

The present study addresses the important issue of nanomaterials safety and their possible impact on human health. Using the TiO_2 nanoparticles (NPs) in a human related *in vitro* toxicity study we show that testing conditions such as dispersion of NPs may have great impact on cytotoxicity and genotoxicity results. In this study we demonstrate that TiO_2 NPs dispersed using one procedure induced DNA strand breaks and oxidized DNA lesions in human and monkey cell lines and thus show positive genotoxic effects. However, using a second dispersion protocol with serum and longer time of sonication no genotoxicity was found. It is therefore important, when assessing the hazard associated with NPs, to establish standard testing procedures and thorough strategies to consider the diverse treatment conditions relevant to possible exposures.

Introduction

Normal sized (coarse and fine) TiO_2 particles are widely used in the production of paints, paper, plastics, welding, rod-coating material, and food colorants.[1] According to the International Agency for Research on Cancer (IARC) classification TiO_2 is possibly carcinogenic to humans (Group 2B).[2] On the other hand nanosized TiO_2 has been reported to interact with cellular membranes and cytoplasm of cultured mammalian cells.[3] TiO_2 nanoparticles (TiO_2 NPs) are used in the pharmaceutical and cosmetics industries.[2] NPs are defined as particles with at least one dimension smaller than 100 nm.[4] Due to the high refractive index, TiO_2 NPs have light-scattering properties and are used for protection against UV exposure (sunscreen products).[5] On the other hand, TiO_2 NPs are also a well-known photocatalyst.[6]

The results of published studies on the genotoxicity of TiO_2 NPs are conflicting, although most of them report TiO_2 NPs to be genotoxic. Gurr *et al.* reported induced DNA oxidation damage in human bronchial epithelial cells BEAS-2B exposed to anatase (10 and 20 nm) TiO_2 NPs in the absence of photoactivation. In contrast, anatase (200 and >200 nm) particles do not induce DNA strand breaks. The form of rutile of size 200 nm, in contrast to the anatase form of size 200 nm, was found to induce DNA oxidation damage. The anatase TiO_2 particles (10 and 200 nm) induced micronuclei, but the anatase (>200 nm) and rutile particles (200 nm) did not increase the number of micronuclei.[6] Rehn *et al.* demonstrated that *in vivo* exposure to TiO_2 P25 (untreated, hydrophilic surface) and TiO_2 T805 (silanized, hydrophobic surface) does not cause persistent DNA damage. They quantified the 8-oxo-7,8-dihydroguanine (8-oxoG) content in the DNA of lung cells 90 days after exposure to TiO_2 P25, TiO_2 T805 and quartz DQ12 (positive control). There was no increase in the levels of 8-oxoG in the TiO_2 NPs exposed Female Wistar rats, compared to the positive control.[7] Wang *et al.* showed that TiO_2 NPs are mutagenic and genotoxic in WIL2-NS human lymphoblastoid cells. Genotoxicity was measured by the cytokinesis block micronucleus (CBMN) assay as a significant increase in the frequency of micronuclei and by the comet assay as an induction of DNA strand breaks. The mutagenicity test (HPRT gene mutation assay) showed a rise in mutant frequency.[1] In the study of Warheit *et al.* TiO_2 NPs were presented as negative in the bacterial reverse mutation test and also induced no chromosomal aberrations in Chinese hamster ovary cells.[8] Eight different tested forms of rutile and anatase ultrafine TiO_2 particles did not increase the frequency of chromosome aberrations in Chinese hamster cells in the absence or presence of UV light and thus do not exhibit photochemical genotoxicity.[5] TiO_2 NPs have a genotoxic effect in human lymphocytes according to both micronucleus and comet assays. Furthermore, they activated p53 DNA damage checkpoint signals.[2] TiO_2 NPs are genotoxic to GFSk-S1 primary fish cells.[3] A positive genotoxic effect of TiO_2 NPs on metabolically competent RTG-2 fish cells was demonstrated only with the comet assay. The CBMN assay has not shown significant induction of micronuclei.[9] The study of Karlsson *et al.* showed that TiO_2 NPs are able to cause DNA damage in human lung epithelial cell line A549.[10] Falck *et al.* examined the genotoxic potential of rutile (silica coated) and anatase form of TiO_2 NPs on human bronchial epithelial (BEAS 2B) cells. Both TiO_2 forms induced DNA damage measured by the comet assay, but only in anatase exposed cells was a slight increase in the number of micronucleated cells observed.[11] Osman *et al.* demonstrated DNA damaging and mutagenic potential of TiO_2 NPs in the HEp-2 cell line.[12] According to the Hackenberg *et al.* study, TiO_2 NPs did not induce any genotoxicity in human peripheral lymphocytes despite NP uptake into the nucleus.[13] No genotoxic effect of TiO_2 NPs was observed on primary human nasal mucosa cells using the comet assay.[14] Genotoxic effects of TiO_2 NP exposure were found as increased frequencies of sister chromatid exchange and micronuclei in Chinese hamster ovary (CHO-K1) cells.[15] A DNA damaging potential of TiO_2 NPs was observed in human epidermal cells (A431). TiO_2 NPs increased DNA oxidation damage (formamidopyrimidine DNA glycosylase, FPG-modified comet assay) and micronucleus formation.[16] TiO_2 NPs were reported to induce clastogenicity and DNA oxidation damage *in vivo* in mice. Orally administrated TiO_2 NPs induced 8-oxoG, γ-H2AX foci, micronuclei and DNA deletions. These results were observed in blood, bone marrow, liver and even embryos.[17] Du *et al.* studied the combined effect of TiO_2 NPs and PbAc on human hepatocytes. After the combined treatment, enhanced 8-oxoG formation (DNA oxidation damage) and OGG1 expression were observed, while after treatment with TiO_2 NPs and PbAc alone, no significant changes were detected.[18]

The published results are inconsistent and difficult to compare, since different parameters and physical and chemical properties can influence results.[19] Additionally there is a lack of information in many publications when it comes to primary and secondary characteristics of NPs as well as dispersion details. As the different testing conditions could affect NP properties and thus also the results of toxicology studies we investigated the role of different dispersion procedures for the identical NPs in the assessment of the genotoxic profile of NPs. TiO_2 NPs used in this study are reference NPs for several laboratories in Europe and for the FP7 project NanoTEST (www.nanotest-fp7.eu) that aims to develop testing protocols for the assessment of toxicity of NPs.[20]

Material and methods

The investigated TiO_2 NPs, an anatase/rutile powder of 21 nm (nominal size), were nanomaterial type NM-105 received from the European Commission—Joint Research Centre (Ispra, Italy). The material corresponds to a selected sample of a nanomaterial produced by Evonik (Essen, Germany) and marketed as Aeroxide TiO_2 P25. Sub-samples of NM-105 were packed under Good Laboratory Practice conditions and preserved under argon in the dark until use. A detailed physical–chemical characterization of TiO_2 NPs has been performed before application to toxicological experiments. In addition, a detailed characterization of secondary characteristics in cultivation media was performed.

Physical and chemical characterization of TiO_2 dry particles

The mean average size of TiO_2 primary particles was determined with a Jeol (Tokyo, Japan) 3010 Transmission Electron Microscope (TEM) operating at 300 kV. The crystal structure was

elucidated by TEM coupled with SAED (Selected Area Electron Diffraction) on the same instrument. The surface area was obtained by the method of Brunauer, Emmett and Teller[21] by nitrogen adsorption on a Micromeritics ASAP 2000 instrument at an adsorption temperature of −196 °C, after pretreating the sample under high vacuum at 300 °C for 2 h. Potential inorganic impurities in the tested nanomaterial were investigated by analysing the provided TiO_2 NPs powder, after acid digestion, by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) on a Perkin-Elmer (Waltham, MA, USA) Optima 5300 DV ICP-OES. Zeta potential was measured by a Nicomp Submicron Particle Sizer Autodilute® Model 380 (Santa Barbara, CA, USA) using the Electrophoretic Light Scattering (ELS) method after sonication of TiO_2 powder in water at different pH values.

Nanoparticles dispersion protocols

Two dispersion protocols (DPs) were used for the treatment. DP1 was developed within the NanoTEST project, DP2 was kindly provided by Prof. F. Marano and Dr S. Boland, University Paris Diderot, France.

TiO_2 NPs dispersion protocol 1 (DP1). Stock solutions at 5 mg ml^{-1} of TiO_2 NPs were prepared fresh each time. To prepare 1 ml of stock solution, 1 ml of 20% foetal bovine serum (FBS) in PBS was added to 5 mg of TiO_2 NPs in a microcentrifuge tube. The dispersion was sonicated with a UP200S probe sonicator by Hielscher Ultrasonic Technology (Teltow, Germany) for 15 min at 100 Watt (cycle: 100%). The dispersion was cooled during sonication with an ice/water bath in order to prevent heating of the dispersion. The resulting stock suspension was added to cell culture medium to achieve a 75 μg cm^{-2} working suspension. Then serial dilutions in the cell culture medium were made to obtain the full range (0.12 to 75 μg cm^{-2}) of NP suspensions. NP-containing media were immediately added to the cells.

TiO_2 NPs dispersion protocol 2 (DP2). Stock solutions of TiO_2 NPs were made by weighing 20 mg of TiO_2 NPs and suspending in 10 ml of culture medium containing 15 mM HEPES buffer without FBS in a 15 ml plastic tube. The suspensions were sonicated using an ultrasonic probe sonicator (Labsonic, Sartorius) for 3 min at 60 W (on ice and water mixture to allow the cooling down of the solution). Within 2 min after sonication and directly after 10 s of vortexing, the solution was divided into 10 microcentrifuge tubes and stored at −20 °C for further use. Immediately before use TiO_2 NPs were thawed, vortexed for 10 s before being immediately sonicated for 1 min (on ice and water mixture) at 60 W, and added to the cell culture medium to achieve a 75 μg cm^{-2} working solution. Then serial dilutions in the cell culture medium were made to obtain the full range (0.12 to 75 μg cm^{-2}) of NP suspensions. NP-containing media were immediately added to the cells.

Size distribution and stability measurements in biological media

Size distribution of dispersed TiO_2 NPs was determined as hydrodynamic diameter by Dynamic Light Scattering (DLS) at 90° detection angle with a Nicomp Submicron Particle Sizer Autodilute® Model 370 (Santa Barbara, CA, USA). The employed instrument can automatically recognize, in the 0.5–6000 nm range, up to three size distributions of particles concurrently present through a patented software algorithm. Appropriate volumes of prepared stock dispersions were added to selected media immediately after preparation and then gently manually shaken. Variation of size distributions after 30 min and after 48 h from addition of dispersions into medium was used as a size stability parameter. All samples were gently homogenized by a Pasteur pipette directly in DLS glass tubes before measurements.

Cell cultivation and incubation with nanoparticles

TK6 human lymphoblast cells (obtained from the European Collection of Cell Cultures (ECACC, cat. no. 95111735)) were maintained in RPMI 1640 (cat. no. R8758, Sigma) culture medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were cultivated at 37 °C in an atmosphere of 5% CO_2 and 100% humidity. Cells were grown in a suspension routinely diluted to 2 × 10^5 to 1 × 10^6 cells per ml. For the experiment, TK6 cells were sub-cultured in a density of 4 × 10^5 cells per ml into 6 well plates with 2 ml of culture medium with freshly dispersed NPs. Cells were exposed to NPs at 5 doses: 0.12, 0.6, 3, 15, and 75 μg cm^{-2} which correspond to 0.57, 2.9, 14.4, 72.0, and 360.2 μg ml^{-1} NPs (calculated for 6 well plates, 2 ml medium). After 2 h, 24 h and 48 h treatments the comet assay, trypan blue exclusion and proliferation activity assays were performed.

Cos-1 monkey kidney fibroblasts (obtained from the European Collection of Cell Cultures, ECACC, cat. no. 88031701) and EUE human embryonic epithelial cells (kindly provided by Professor Angelo Abbondandolo, Pisa, Italy) were cultivated in DMEM medium (Dulbecco's Modified Eagle's Medium, cat. no. D6046, Sigma) containing 10% FBS (cat. no. 26140-079, Invitrogen), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (cat. no. 15140122, Invitrogen) in a humidified atmosphere at 37 °C and 5% CO_2. The cells were seeded in 6 well plates at a density of 5 × 10^4 cells per well, and grown for ~48 h to reach the required 70% confluence. Before exposure the culture medium was removed. Freshly dispersed NPs were diluted to 2 ml with medium (total volume) and vortexed for ~15 seconds before adding the complete mix of NPs and medium to the cells. After 2 or 24 h NP exposure the cells were used for the comet assay and plating efficiency assay.

Trypan blue exclusion assay

For the cell viability assay TK6 cells were exposed to NPs for 2 h and 24 h. TK6 cells were disaggregated in the medium after the exposure. 10 μl of cell suspension was mixed with 10 μl trypan blue (0.4%) and the cell viability (percentage of stained cells) was measured using a Countess™ Automated Cell Counter (Invitrogen). Cell viability was determined according to the formula: viability (%) = 100 − (number of dead cells/total number of cells) × 100.

Proliferation activity of the cells exposed to NPs

TK6 cells (growing in suspension) exposed for 2 or 24 h to TiO_2 NPs (dispersed using two different dispersion protocols, DP1

and DP2) at 5 doses were disaggregated and counted using a Countess™ Automated Cell Counter (Invitrogen) at 24 h intervals for 2–3 days.

Cos-1 and EUE cells, after 24 h of treatment with TiO_2 NPs, were washed with PBS, detached using trypsin–EDTA, resuspended in culture medium, counted using a Countess™ Automated Cell Counter (Invitrogen), and seeded at a concentration of 2×10^4 cells per well. Counts were repeated at 24 h intervals for 2–3 days during cultivation to assess proliferation activity.

Plating efficiency (PE)

Cos-1 cells at the required confluence level were exposed to NPs dispersed in fresh medium at 5 concentrations for 24 h. After the exposure the medium with NPs was removed, cells were washed with PBS, and trypsin–EDTA was added to detach the cells. Cells were then resuspended in culture medium and counted. 200 cells per well (6 well plates) in 5 replicates were inoculated for every exposure concentration, and cultivated for approximately 10 days allowing cells to form colonies. Colonies were stained with 1% methylene blue in 20 μl per well for 30 min, rinsed with water and counted. PE was calculated according to the formula: PE (%) = (number of colonies formed/number of cells plated) × 100.

Alkaline comet assay and FPG-modified comet assay

The TK6, Cos-1 and EUE cells exposed to TiO_2 NPs were used for the comet assay after 2 and 24 h. After treatment cells were washed with PBS, detached from the culture plates using trypsin–EDTA and re-suspended in culture medium. TK6 cells (growing in suspension culture) were resuspended. Cells were distributed into microcentrifuge tubes and centrifuged (200*g*, 3 min, 4 °C). The supernatant was removed and cells were dispersed in the small volume of medium remaining. The cells were embedded in 1% low melting point (LMP) agarose in PBS at 37 °C, placed on a glass microscope slide, and covered with glass cover slips. Slides were precoated with 0.5% normal melting point agarose in H_2O before use. Two gels per slide with cells were prepared, placed for 5 min at 4 °C and immersed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris–HCl, pH 10 with 1% Triton X-100 added immediately before use) for 1 h at 4 °C. During lysis cell membranes, cytoplasm and most chromatin proteins are removed, leaving the free nuclear DNA in the agarose gel.

For FPG treatment, slides were washed in 3 changes of cold (4 °C) FPG reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg ml^{-1} bovine serum albumin, pH adjusted to 8.0 with KOH) in a staining jar, for 5 min each. 50 μl of FPG solution (final dilution of the batch 2004: 1/3000) or reaction buffer alone (as a negative control) was added onto the gel, and covered with a square cut of Parafilm. Slides were incubated in a moist box for 30 min at 37 °C, put for 5 min at 4 °C and Parafilm removed. FPG was kindly provided by Professor Andrew Collins (Department of Nutrition, University of Oslo, Norway). After lysis or enzyme incubation (FPG-modified comet assay), the slides were placed in a horizontal electrophoresis tank, immersed in cold (4 °C) electrophoresis solution (0.3 M NaOH; 1 mM EDTA) for 20 min and subjected to electrophoresis at 25 V (around 300 mA) for 20 min. After neutralization for 10 min in PBS, followed by 10 min in water, slides were dried at room temperature. Samples were stained with 20 μl of a 1 μg ml^{-1} 4′,6-diamidine-2-phenylindol dihydrochloride (DAPI), covered with a cover slip and viewed by a fluorescence microscope (Leica DMI 6000 B) and a CCD camera. Quantification was made by image analysis system Comet assay IV (Perceptive Instruments Ltd.) by scoring 100 comets per sample. Positive controls: cells exposed to 50 μmol dm^{-3} H_2O_2 for 5 min at 4 °C; cells exposed to photosensitiser Ro 19-8022 (1 μM in PBS; kindly provided by LaRoche) and visible light (250 W, 33 cm distance) for 4 min on ice (for FPG enzyme modified comet assay). As negative control unexposed cells cultivated in culture (treatment medium) were used.

Statistical analysis

Student's *t*-test was used for statistical analysis. Data are presented as mean values ± standard deviation. *P*-Values interpret statistically significant results: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

Nanoparticle characterization

The TiO_2 NPs were characterized in terms of physical and chemical properties before performing toxicological experiments in order to confirm the specifications declared by the manufacturer and to correlate them with their toxicological behaviour. The results are summarized in Table 1. In Fig. 1 the zeta-potential *vs.* pH of the TiO_2 NP batch is reported. In Fig. 2 representative TEM images of selected NPs are shown. In addition, a detailed investigation of NP behaviour in two biological media (DMEM and RPMI) using two different DPs is summarized in Table 2, where average size distributions of TiO_2 stock dispersions 1 and 2 added to applied biological media are presented. The DP1 procedure gives more stable (up to 2 days) bimodal dispersion with two peaks more or less in the nanosized range while DP2 results in large agglomerates and less stable dispersion (Table 2). Culture media did not influence the final dispersion.

Effects of TiO_2 NPs dispersed according to protocols 1 and 2 on cytotoxicity and cell proliferation

TiO_2 NPs dispersed according to protocols 1 and 2 (DP1 and DP2) were investigated for their cytotoxic effects on TK6, Cos-1

Table 1 Summary of primary physical and chemical properties of selected TiO_2 NPs

Primary physical–chemical characteristics	
Appearance	White ultrafine dry powder
Shape of particles	Irregular/ellipsoidal
Particle size	15–60 nm
Crystal structure	Rutile/anatase
Surface area/m^2 g^{-1}	61
Pore volume	0.13 mL g^{-1}
Pore size distribution	Bimodal, 4–8 nm and 20–70 nm
Zeta-potential at pH 7/mV	−30.2
Chemical composition of particles	Ti, O
Purity of particles	>99%
Surface chemistry	Uncoated
Impurities of concern	Co (920 ppm), Fe (16 ppm)

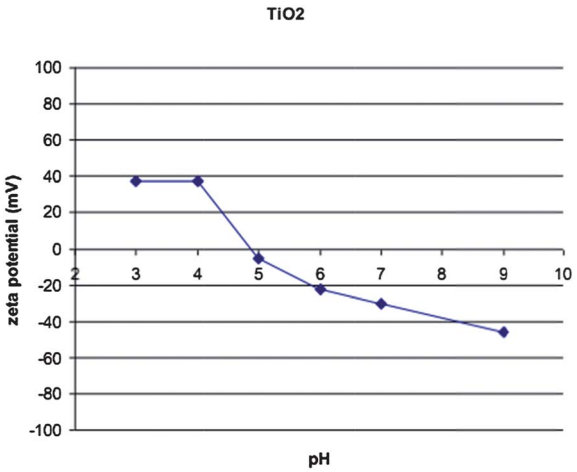

Fig. 1 Zeta potential of TiO_2 nanopowder in water at different pH values.

and EUE cells. The cells were exposed to TiO_2 NPs at 0.12, 0.6, 3, 15, and 75 μg cm^{-2} and trypan blue exclusion, plating efficiency and proliferation assays were performed. NP concentrations which had effects >20% loss in cell survival compared to the negative control were considered as cytotoxic.

As shown in Fig. 3A and B, 4A and B and 5A and B, TiO_2 NPs prepared according to DP1 and DP2 did not affect trypan blue exclusion or proliferation activity of TK6 cells in the tested concentration range and at either of the time-points.

Results in Fig. 6B show that 24 h treatment with TiO_2 NPs dispersed according to DP2 at concentrations 15 and 75 μg cm^{-2} inhibited plating efficiency of Cos-1 cells. Similarly, the proliferation activity of Cos-1 cells was slightly decreased after exposure to 15 μg cm^{-2} TiO_2 NPs and strongly inhibited at an NP concentration of 75 μg cm^{-2} as shown in Fig. 3C.

TiO_2 NPs dispersed according to DP1 in the studied concentration range did not inhibit proliferation activity of EUE cells after 24 h exposure, while treatment with DP2-dispersed TiO_2 NPs caused slight decrease in proliferation of cells at the two highest concentrations (6 and 30 μg cm^{-2}) as is presented in Fig. 3D and E.

Effects of TiO_2 NPs dispersed according to protocols 1 and 2 on the level of DNA strand breaks and oxidized DNA lesions

The alkaline comet assay with and without lesion-specific glycosylase FPG was used to assess the DNA damaging potential of TiO_2 NPs in TK6, Cos-1 and EUE cells. DNA damage is expressed as strand breaks (SBs) and DNA oxidation damage as FPG sensitive sites (Net FPG), representing altered purines. As positive controls, DNA damaging agent H_2O_2 was used for DNA strand breaks (giving an average value of 20.4% tail intensity) and photosensitizer Ro 19-8022 plus visible light for oxidized DNA lesions (FPG sites) (32.9% tail intensity).

According to the comet assay results presented in Fig. 4A and B, TiO_2 NPs dispersed using DP1 do not cause any significant increase in the DNA damage level compared to negative unexposed control after 2 h and 24 h treatment of TK6 cells in test conditions. As presented in Fig. 5A, we did not find significant effects of TiO_2 NPs prepared according to DP2 on the induction of DNA strand breaks in TK6 cells after 2 h exposure. But using the FPG modified comet assay, applied to assess oxidized DNA

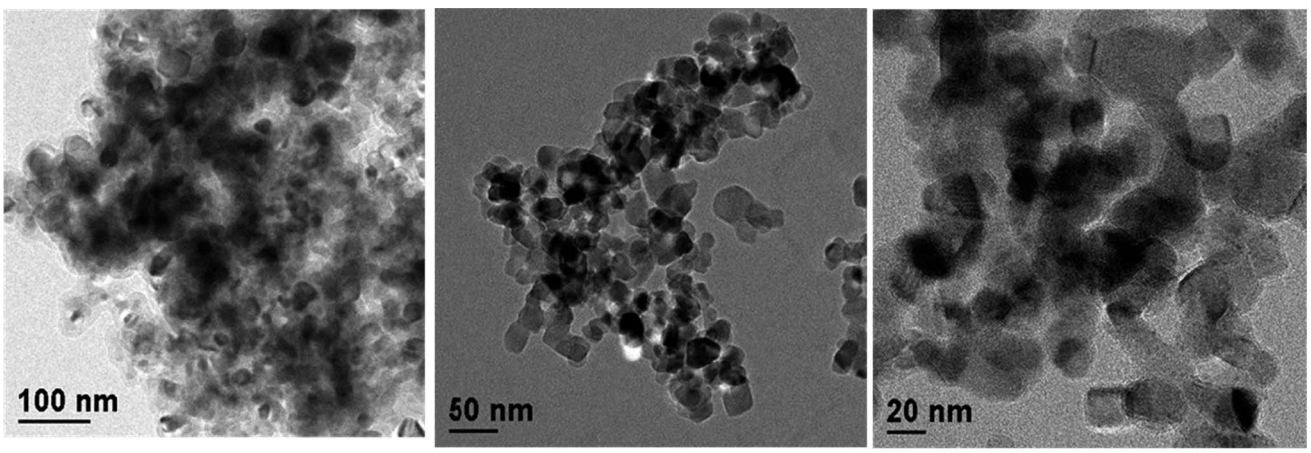

Fig. 2 Representative Transmission Electron Microscopy (TEM) images of the investigated TiO_2 nanopowder at different enlargements.

Table 2 Average hydrodynamic diameters determined by Dynamic Light Scattering (DLS) of TiO_2 stock dispersions (DP1 and DP2) in two culture media

Culture medium (nominal TiO_2 NPs conc.: 0.25 mg ml^{-1})	TiO_2 stock dispersion (DP1)	TiO_2 stock dispersion (DP2)
RPMI-1640 + 10% FBS	Bimodal distribution, 102 (±15) nm and 285 (±67) nm	779 (±382) nm
Size stability	Stable ≈ 2 days	Agglomerated
DMEM + 10% FBS	Bimodal distribution, 112 (±20) nm and 296 (±55) nm	752 (±397) nm
Size stability	Stable ≈ 2 days	Agglomerated

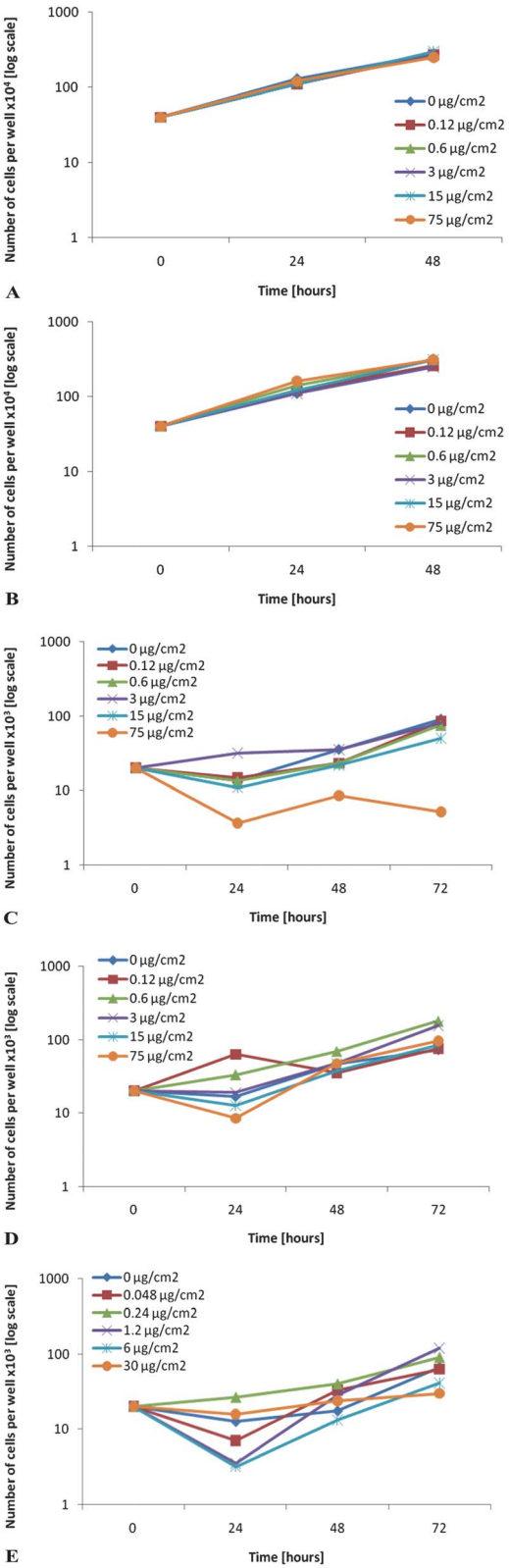

Fig. 3 (A) Proliferation activity of TK6 cells exposed to TiO_2 NPs using the DP1 dispersion protocol. A number of cells were counted at 0, 24 and 48 h. (B) Proliferation activity of TK6 cells exposed to TiO_2 NPs using the DP2 dispersion protocol. A number of cells were counted at 0, 24 and 48 h. (C) Proliferation activity of Cos-1 cells exposed 24 h to TiO_2 NPs using the DP2 dispersion protocol. A number of cells were counted in 24 h intervals. (D) Proliferation activity of EUE cells exposed 24 h to TiO_2 NPs using the DP1 dispersion protocol. A number of cells were counted in 24 h intervals. (E) Proliferation activity of EUE cells exposed 24 h to TiO_2 NPs using the DP2 dispersion protocol. A number of cells were counted in 24 h intervals.

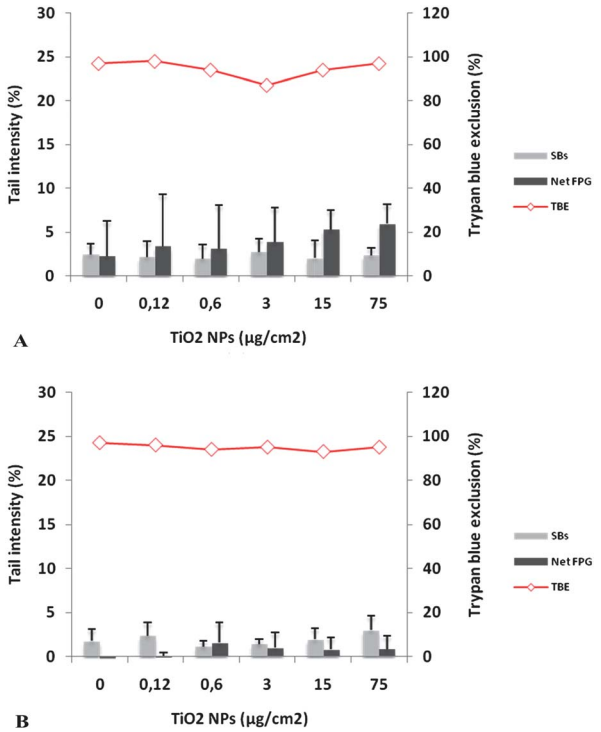

Fig. 4 DNA damage measured by the comet assay after 2 h exposure (A) and 24 h exposure (B) of TK6 cells to TiO_2 NPs dispersed using DP1. Strand breaks (SBs) were measured using an alkaline version of the comet assay (CA). Oxidized purines were detected with modified CA using formamidopyrimidine glycosylase (FPG), and are expressed as Net FPG (FPG-sensitive sites representing oxidized purines). Cytotoxicity was measured by trypan blue exclusion assay (TBE). Data are presented as mean values ± standard deviation.

lesions, we detected a statistically significant increase in the level of FPG sites after 2 h exposure of TK6 cells to the highest concentration (75 µg cm^{-2}) of TiO_2 NPs (DP2). 24 h treatment with TiO_2 NPs (DP2) did not significantly increase strand breaks or oxidized DNA lesions in TK6 cells compared to untreated control (Fig. 5B).

TiO_2 NPs dispersed according to DP2 significantly increased the levels of SBs in Cos-1 cells after 2 h and 24 h exposure at the highest concentration (75 µg cm^{-2}) (Fig. 6A and B). However, induction of DNA damage at this dose could have been due to the cytotoxicity observed with the plating efficiency and proliferation activity assays. We observed no induction of DNA oxidation damage, except at 3 µg cm^{-2} after 24 h treatment, when there was a slight but significant induction of FPG sites.

Using the comet assay, 24 h exposure of EUE cells to TiO_2 NPs dispersed according to DP1 displayed no increase in DNA damage over the whole concentration range (Fig. 7A), while TiO_2 NPs dispersed according to DP2 significantly induced the level of SBs at 75 µg cm^{-2} (Fig. 7B).

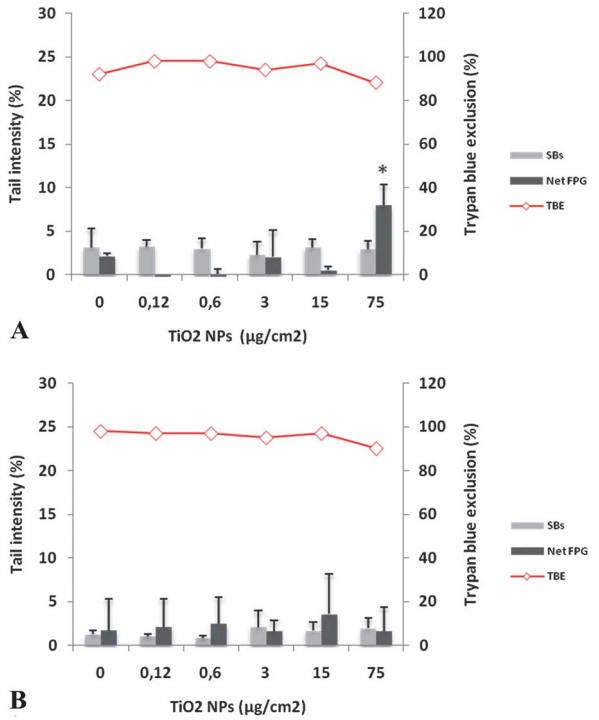

Fig. 5 DNA damage measured by the comet assay after 2 h exposure (A) and 24 h exposure (B) of TK6 cells to TiO_2 NPs dispersed using DP2. Strand breaks (SBs) were measured using an alkaline version of the comet assay (CA). Oxidized purines were detected with modified CA using formamidopyrimidine glycosylase (FPG), and are expressed as Net FPG (FPG-sensitive sites representing oxidized purines). Cytotoxicity was measured by the trypan blue exclusion assay (TBE). Data are presented as mean values ± standard deviation.

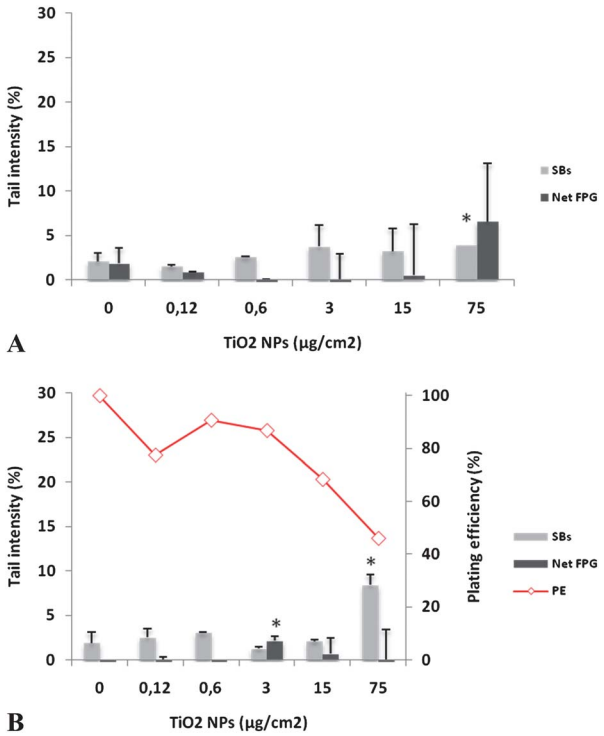

Fig. 6 DNA damage measured by the comet assay after 2 h exposure (A) and 24 h exposure (B) of Cos-1 cells to TiO_2 NPs dispersed using DP2. DNA strand breaks (SBs) were measured using an alkaline version of the comet assay (CA). Oxidized purines were detected with modified CA using formamidopyrimidine glycosylase (FPG) and are expressed as Net FPG (FPG-sensitive sites representing oxidized purines). Data are presented as mean values ± standard deviation. Representative experiment.

Discussion

The published results on TiO_2 NPs cytotoxicity and genotoxicity are inconsistent and difficult to compare as information on physical and chemical properties as well as dispersion details are often missing. In addition to small size other properties such as size distribution, crystal form, surface area, purity, method of dispersion, coating type, different toxicity assays, cell types, cultivation and exposure conditions can influence results. Unfortunately, there is still a lack of systematic studies that would allow comparison of the NP specific effect with their physical and chemical properties. These properties may play an important role in the impact of NPs on cytotoxicity and genotoxicity. Different testing conditions can also modulate NP properties and thus influence the results, such as stability of NPs in cell culture media[19] or state of agglomeration.[22] TiO_2 NPs are prone to form agglomerates in solutions[17] and therefore dispersion in testing medium is crucial.

When comparing data published on the TiO_2 NP, the conflicting results might partially relate to the presence or absence of proteins (either FBS or BSA) and sonication in the TiO_2 NP dispersion protocol. Negative genotoxic results observed for instance in Hackenberg *et al.* used BSA and sonication in the dispersion protocol.[13,14] However, in the study of Warheit *et al.* which did not use any proteins for the NP dispersion negative results were also observed.[8] In this study there is a need to consider also whether genotoxicity assays used (Ames test and chromosome aberration assay) are suitable for the assessment of genotoxicity of NPs since most of the available literature data with these tests show negative results.[23–28]

In our study two different dispersion protocols were compared: one with serum in stock solution and one without in order to investigate whether the dispersion procedure and dispersion components could influence NP cytotoxicity and genotoxicity. In addition to primary characteristics of TiO_2 NPs, we carried out a detailed secondary characterization of NPs dispersed in two cell culture media (DMEM and RPMI both with 10% FBS) using two dispersion protocols. Our results show that dispersion was not influenced by the type of culture medium. The level of agglomeration/aggregation of NPs and size distribution depends on the dispersion procedure and the use of serum in stock solution. The protocol DP1 using FBS gave a relatively stable dispersion of TiO_2 NPs, while with the second protocol DP2, rapid formation of TiO_2 NP agglomerates in the testing medium was obtained. In most of the studies with positive results on TiO_2 NPs genotoxicity, no serum or BSA in combination with sonication was used (or at least they were not listed in methods).[3,6,12,15] However, some of the studies which show positive genotoxicity results used proteins and sonication.[1,16] Although the duration of sonication and content of proteins need to be considered in these studies. The amount of proteins in dispersion might be also important (to be sufficient to cover NPs

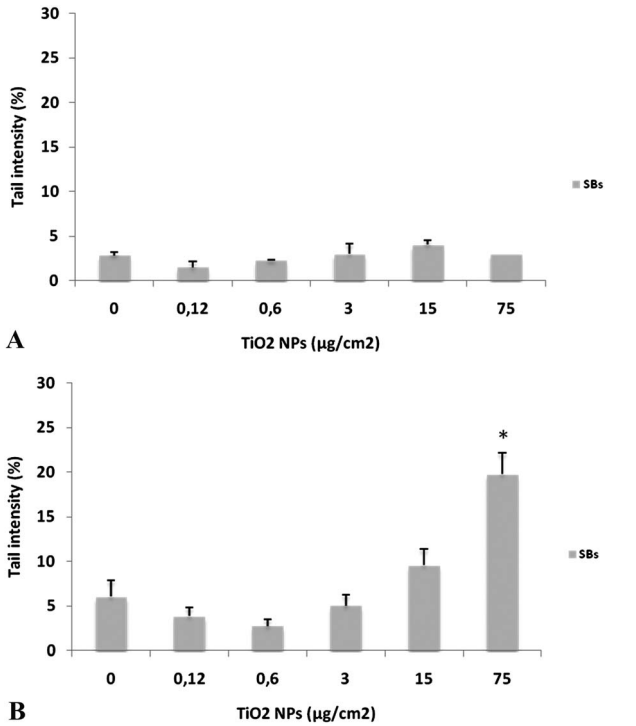

Fig. 7 (A) DNA damage measured by the comet assay after 24 h exposure of EUE cells to TiO_2 NPs dispersed using DP1. DNA strand breaks (SBs) were measured using an alkaline version of the comet assay (CA). Data are presented as mean values ± standard deviation. Representative experiment. (B) DNA damage measured by the comet assay after 24 h exposure of EUE cells to TiO_2 NPs dispersed using DP2. DNA strand breaks (SBs) were measured using an alkaline version of the comet assay (CA). Data are presented as mean values ± standard deviation. Representative experiment.

and form protein corona). In the DP1 protocol we used 20% FBS to give stable TiO_2 NPs dispersion, while the above-mentioned studies used 5% or 10% FBS in the dispersions. Our results show that the presence of serum in stock dispersion medium and the length of sonication may modify the surface of NPs and affect their behaviour. While the DP1 procedure (dispersion in the presence of serum and 15 min sonication) resulted in smaller particles in the nanosized range and was stable for several hours, DP2 (without serum and sonication only 3 min) produced unstable dispersion (stable only a few min) with large agglomerates. Serum proteins adhere to their surface forming a protein corona and so help them to disperse in solution.[29–31]

The fact that different degrees of agglomeration/aggregation of NPs may cause different biologic responses was already addressed by Wick *et al.*[32] in a carbon nanotube cytotoxicity study. The TiO_2 NPs from the provider and the same batch as used in our study were used to examine how the agglomeration/aggregation state (using two dispersion protocols) may affect the kinetics of cellular binding/uptake and ability to induce cytotoxic responses in THP1, HepG2 and A549 cells.[33] Cellular binding/uptake was assessed by SEM/TEM and by the SSC flow cytometry, metabolic activation and cell death measurements by the MTT-test and the propidium iodide assay. The authors show that TiO_2 NPs were efficiently taken up by the cells and there was no difference between dispersion protocols used. The magnitude of the decreasing metabolic activation and increasing cell death depended on the agglomeration/aggregation state of particles, their size, time-point and cell type. To examine the possible impact of different degrees of agglomeration on the cell response, we measured DNA strand breaks and base DNA oxidation lesions, especially 8-oxoG by the comet assay modified with specific repair enzyme FPG. Proliferation activity, plating efficiency and trypan blue exclusion assays were used to evaluate the cytotoxic effect of TiO_2 NPs. Two human cell lines, adherent (EUE) and suspension (TK6), and one Cos-1 monkey cell line were used. We found that DP1 gave stable dispersion in both culture media for two days and smaller agglomerates than when using protocol DP2, most likely because of the presence of serum and the formation of protein corona. In contrast, DP2 resulted in less stability and formation of larger agglomerates. Interestingly, in spite of larger agglomerates and less stable dispersion, TiO_2 NPs dispersed using DP2 (no serum in stock solution and sonication only 3 min) induced slight cytotoxic and clear genotoxic effects at higher concentrations of TiO_2 NPs. It is possible that larger agglomerates might be less stable and thus NPs can be released and reach cells as individual NPs. Using DP2, without serum, the surface of NPs in agglomerates might not be covered by a protein corona and thus the effect can be more pronounced. We also need to note that TiO_2 NPs dispersed according to DP2 (not stable) precipitate quickly at the bottom of the cell culture dish, so that the real amount of NPs to which cells are exposed could be actually greater than in the case of NPs dispersed in culture medium (DP1). Thus the precipitation of NPs is important to consider while performing toxicology testing. Our results show that TiO_2 NPs are not cytotoxic and do not induce DNA strand breaks (measured with the comet assay) in TK6 lymphoblastoid cells over the whole concentration range tested. However, the FPG-modified comet assay showed different results dependent on the DP by which the NPs are dispersed in cell culture medium. TiO_2 NPs dispersed according to DP2, at a concentration of 75 µg cm^{-2} that was found to be non-cytotoxic, displayed significant induction of oxidized DNA lesions, while the TiO_2 NPs dispersed according to DP1 had no effect on genotoxicity under the same conditions. In general smaller NPs are considered to be more toxic because they can more easily enter the cell. Interestingly, DP2 which form agglomerates caused DNA damage, in contrast to DP1 dispersed NPs. However, NP aggregates could mechanically damage the cell and as shown by TEM observations,[15] aggregates can also deform the nucleus on entry into the cell. The study of Lankoff *et al.* shows that TiO_2 NPs in smaller as well as larger agglomerates were taken up by all cell types studied. No significant differences between protocols 1 and 2 were observed.[33]

In our study we investigated a mixture of anatase/rutile TiO_2 NPs. These two forms of TiO_2 NPs may have different genotoxic potential as it was shown by Falck *et al.* where anatase was more potent than rutile.[11] However, some studies on anatase show no genotoxicity.[34] Generally, results published on the comet assay report mostly a positive genotoxic effect of both forms as well as their mixtures.[10]

It is questionable by which mechanism aggregates of TiO_2 NPs could cause oxidative damage to DNA. As within the 2 h treatment they cannot get direct access to DNA since they are too large to enter the nucleus *via* nuclear pores, their mechanism of

action is probably indirect. A possible reason that we did not find any DNA oxidation damage after 24 h treatment of TK6 cells is that the DNA lesions had been repaired. It is debatable which dispersion protocol is more appropriate for assessment of NP toxic effects. From results obtained in this study we suggest both conditions to be incorporated into testing strategies, since the results could differ. More ideally dispersed TiO_2 NPs could be used to study the effect of particular NPs (not aggregates) on the cell response, though they have a protein corona on their surface and thus TiO_2 NPs themselves might not come to direct contact with cells (the toxic effect of NPs could be hidden behind the protein corona). However, this situation is likely to occur in the human body as after the NPs enter the body they are coated by cellular proteins. In the case of exposure of humans to NPs present in the environment, NPs are not coated with a protein corona and they are more likely to occur in agglomerates/aggregates before they enter the body. We show differences in the levels of DNA oxidation damage, depending on the dispersion method used. TiO_2 NPs in large agglomerates induced DNA damage in all three cell lines. However, only strand breaks were found in EUE cells. TiO_2 (DP2) induced oxidized DNA lesions in Cos-1 and TK6 cells. Our results are in agreement with those of Bhattacharya *et al.* who studied the ability of the TiO_2 NPs to induce genotoxicity and genomic damage caused by oxidative stress (8-oxoG) in IMR 90 (human bronchial fibroblasts) and BEAS-2B cells. TiO_2 NPs did not induce DNA-breakage measured by the comet assay in both cell lines. A high level of DNA adducts (8-oxoG) was observed in IMR-90 cells exposed to TiO_2 NPs.[34] Similarly, Reeves *et al.* found an increase in levels of FPG-sensitive sites after exposure of the primary fish cell line to TiO_2 NPs. Since the level of endonuclease-III-sensitive sites (oxidized pyrimidines) did not increase in contrast to FPG-sensitive sites (8-oxoG and also possible formamidopyrimidines), as the main substrates for FPG, they were indicated to be the most likely DNA lesions produced by TiO_2-mediated oxidative stress.[3] Shukla *et al.* also observed the DNA-damaging potential of TiO_2 NPs in human epidermal cells (A431). TiO_2 NPs increased DNA oxidation damage (measured with FPG-modified comet assay) and micronucleus formation. The strong correlation between DNA oxidation damage and ROS generation supports our finding that ROS might be involved in the mechanism of genotoxicity.[16] Our results also showed that induction of DNA damage by TiO_2 is dependent on the dispersion protocol, state of aggregation/agglomeration, concentration used, time of treatment and cell type.

There is still a lack of guidelines and standard protocols for human related toxicity as well as ecotoxicity. It is becoming clear that toxicity tests presently used in hazard assessment of chemicals need to be adapted to fit the unique features of the nano-sized material.[20,35] Minimal characterization needs to be implemented into test procedures.[36,37] Our results show that several dispersion and testing conditions might also be needed to assess NP toxicity.

Conclusions

Different NP dispersion procedures used in toxicity testing result in different agglomeration states and may have considerable impact on final cytotoxicity and genotoxicity results. Here we demonstrate that TiO_2 NPs dispersed using one procedure induced strand breaks and oxidized DNA lesions in human and monkey cell lines and thus show positive genotoxic effects. However, using a second dispersion protocol no genotoxicity was found. It is therefore important, when assessing the hazard associated with NPs, to establish standard testing procedures and thorough strategies to consider the diverse conditions relevant to real exposure.

Acknowledgements

The work was supported by EC FP7 [Health-2007-1.3-4, contract no: 201335] and by Polish-Norwegian Research Fund [PRNF/122-A I-1/07]. The technical support by Riccardo Cossi (Qi srl, Pomezia, Italy) during DLS measurements is thankfully acknowledged. The authors would also like to thank D. Cristofori, V. Trevisan and Flavia Visin (University Ca' Foscari Venice) for TEM/SAED investigations, surface area (BET) measurements and ICP-OES analysis, respectively. We thank Prof. Francelyne Marano and Dr Sonja Boland for providing the dispersion protocol and Prof. Andrew Collins for his comments and English editions.

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