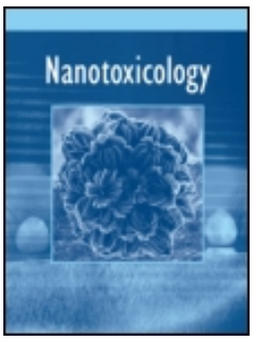

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Experimental evidence of false-positive Comet test results due to TiO_2 particle – assay interactions

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Abstract

We have studied the genotoxicity of TiO_2 particles with a Comet assay on a unicellular organism, *Tetrahymena thermophila*. Exposure to bulk- or nano-TiO_2 of free cells, cells embedded in gel or nuclei embedded in gel, all resulted in a positive Comet assay result but this outcome could not be confirmed by cytotoxicity measures such as lipid peroxidation, elevated reactive oxygen species or cell membrane composition. Published reports state that in the absence of cytotoxicity, nano- and bulk-TiO_2 genotoxicity do not occur directly, and a possible explanation of our Comet assay results is that they are false positives resulting from *post festum* exposure interactions between particles and DNA. We suggest that before Comet assay is used for nanoparticle genotoxicity testing, evidence for the possibility of *post festum* exposure interactions should be considered. The *acellular* Comet test described in this report can be used for this purpose.

Keywords: *Tetrahymena thermophila*, DNA damage, nanoparticles, nanotoxicity

Introduction

Genotoxicity has been defined by the International Conference of Harmonization in an ICH-Guideline as deleterious change in the genetic material induced by any mechanism. Damage to DNA results in cellular dysfunction and may therefore initiate and promote mutagenesis and carcinogenesis, or impact fertility (Sathya et al. 2010). Because of this, data on genotoxicity are of great importance in regulatory health risk assessment.

Genotoxicity of nanoparticles (NP) has frequently been documented (Sathya et al. 2010) (Landsiedel et al. 2010; Karlsson 2010) and the mechanisms of this genotoxicity include direct primary genotoxicity driven by direct interaction of NPs with DNA (Donaldson et al. 2010) and indirect primary genotoxicity resulting from oxidative stress (Nel et al. 2006). Oxidative stress occurs when NPs are transported into the nucleus (Chen & von Mikecz 2005) (AshaRani et al. 2009) or when the nuclear membrane breaks down during mitosis (Karlsson 2010). An example of an indirect mechanism is enhancement of the permeability of the lysosomal membrane, leading to release of DNase, which, transported to the nucleus, can degrade DNA (Banasik et al. 2005). Secondary indirect mechanisms of nanoparticle genotoxicity are associated with inflammation (Trouiller et al. 2009).

With the advent of nanotechnology, it is essential to define a reliable test system with which the genotoxic potential of engineered NPs can be assessed (Warheit & Donner 2010; Gonzalez et al. 2011). Guidelines provided by the Organisation for Economic Cooperation and Development (OECD) include *in vitro* genotoxicity testing, but these tests are designed basically for water-soluble chemicals and so may not be suited to the testing of the genotoxicity of NPs. Nanoparticles interfere with test media, modifying the biological potential of the NPs, and they may also interact with the test system, affecting the test results (Sathya et al. 2010); (Greim & Norppa 2010). In an attempt to clarify this issue, the OECD has established projects designed to evaluate the relevance and reproducibility of genotoxicity assays (see (Warheit & Donner 2010); Stone et al. (2009)) have shown the importance when assessing direct primary genotoxicity of accurate distinction of artefacts and the possible interaction of test components with nanoparticles remaining in the test system after exposure. Residual NPs may come into contact with nuclear DNA during tests affecting the test, and this may also happen when NPs present inside cells in the cellular lysosomes or food vacuoles are released during the tests.

In recent research of genotoxicity and nanoparticles, the Comet assay has been one of the most frequently used tests (Landsiedel et al. 2009) (Karlsson 2010). Recently, Karlsson (2010) reviewed 46 papers dealing with the genotoxicity of NPs by the Comet assay and concluded that majority of

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the NPs tested caused DNA strand breaks. However, the possibility of interaction of NPs with the chemicals used in the assay was cited and the use of additional methods, distinct from the Comet assay, was suggested for the measurement of DNA damage. Further mutagenicity studies have also been recommended. Landsiedel et al. (2009) suggested the use of a battery of standardised genotoxicity tests covering a wide variety of potential mechanisms and suggested that at least two genotoxicity tests should always be implemented.

At present, there are four techniques in common use for *in vitro* testing the genotoxicity of nanoparticles. These are the Ames test, the Chromosomal Aberration Test, the Comet assay and the Micronucleus test. Of these, the Comet assay is the most popular because (1) it is sensitive and capable of detecting low levels of DNA damage, (2) it requires only small numbers of cells per sample, (3) it is relatively inexpensive and (4) it requires relatively small amounts of test substance (Tice et al. 2000). Among the limitations and disadvantages of the Comet assay is its failure to detect: (1) aneugenic effects, (2) epigenetic mechanisms of DNA damage (Dhawan et al. 2009) and (3) fixed mutations (Stone et al. 2009). There are also some serious obstacles to the use of the Comet assay for NP genotoxicity studies. Karlsson (2010), for example, has shown the presence of nanoparticles (nano-TiO_2 and nano-CuO), in heads of the comets in the gels, while intracellular localisation of particles investigated by TEM did not reveal particles in cell nuclei. The possibility of post-exposure particle DNA interactions was also discussed by Lin et al. (Lin et al. 2009), studying the genotoxicity of Ge nanoparticles by the Comet assay. They noted a statistically higher level of DNA damage in exposed cells when compared with control cells and speculated that since nanoparticles of Ge readily adhere to cell surfaces, nanoparticles in or attached to the cells caused the damage during the assay process.

The features that motivated the selection of *Tetrahymena thermophila* as a model organism for this study are as follows: (1) it is a one-cell eukaryotic organism. Thus, the data obtained by Comet assay correspond to the impact of TiO_2 on whole-organism DNA, and in summary the effects are measured on the genome of the entire cell population. (2) Its short generation time and its axenic culture are especially advantageous for studying genotoxicity. (3) As protists have highly developed systems for internalisation of nanoscale (100 nm or less) and microscale (100 – 100,000 nm) particles (Frankel 2000), they are very good model organisms for nanotoxicology (Holbrook et al. 2008) (Kahru et al. 2008). (4) It has been used in toxicology for decades as a useful model organism for cellular and molecular biologists as well as for environmental research (Sauvant et al. 1999; Gutiérrez et al. 2003).

The aim of the present study was to provide experimental evidence on the possibility that NPs interact with the DNA *post festum*, during a Comet assay. We used a unicellular model organism *T thermophila* to assess genotoxicity by a Comet assay and cytotoxicity by conventional markers. In our study, three exposure scenarios were used: (1) *in vivo* exposure: *T. thermophila* was incubated in a suspension of particles – both nanoparticles and bulk-TiO_2; (2) *in vitro* exposure: *T. thermophila* was embedded in gels that were incubated in a suspension of particles; (3) *acellular* exposure: only nuclei were embedded in gels and the gels with embedded nuclei were incubated in a suspension of particles. We chose to examine nano-TiO_2 particles for which a substantial amount of genotoxicity data already exists. (Trouiller et al. 2009) suggested that DNA damage results not from direct primary effects of nano-TiO_2 but rather from reactive oxygen species (ROS) generation and is therefore a primary indirect effect. Very same was confirmed also by Petkovic et al. (2011a, b) (Petkovic et al. 2011a) (Petkovic et al. 2011b). Consequently, we hypothesise that *in vitro* exposure (cells embedded in gel) and *acellular* exposure of only nuclei to nano-TiO_2 would fail to produce a positive result in a Comet assay since ROS generation, a primary indirect effect, would be absent. If *in vitro* and *acellular* exposure were to lead to a positive Comet assay, this would suggest that particles could damage DNA during the tests, producing the positive Comet test result. In such cases, the use of Comet assay would have to be critically reconsidered.

Materials and methods

Chemicals

Unless otherwise specified, reagents were purchased from Sigma Aldrich Co (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Biolife (Milan, Italy). TiO_2 nanoparticles with 99.7% purity were supplied in the form of a powder.

T. thermophila growth conditions

Axenic cultures of *T. thermophila* from the Protoxkit FTM (MicroBioTests Inc.) were grown for 24 h in the dark at 25 C in a semidefined proteose-peptone-based "rich" medium (RM) (Schultz 1997). The cell density obtained after incubation in these culture conditions was approximately 10^5 cells/ml.

Exposure conditions

The cells were harvested by 3 min centrifugation at 60 rcf. Cells were washed and resuspended in a "poor" medium (PM), which consisted of the semidefined proteose-peptone-based medium used by Schultz (Schultz 1997), but lacking yeast extract and bacteriological peptone. The pH of the medium was adjusted to 7.4 and temperature was maintained at 25 C for the entire experiment. All experiments were performed in 100 ml batch cultures that were maintained in Erlenmeyer flasks and aerated by shaking at 90 rpm in an incubator in the dark.

After 1 h in the PM, cells were exposed to bulk- or nano-TiO_2. The final concentration of particles in the medium, either bulk- or nano-, was 0.1 and 100 μg/ml. Following the addition of TiO_2, *T. thermophila* cultures were incubated at 25 C for 4 h. For each concentration of bulk- or nano-TiO_2, three independent assays were carried out. A supplementary set of three replicates without TiO_2 was set up as a control for each assay. After 4-h treatment with TiO_2 bulk- or nanoparticles, 15 ml of cell suspension was harvested for the purpose of cellular fatty acid composition analysis by gas chromatography.

Bulk- and nano-TiO_2 tested suspension

Aqueous dispersions of nanoparticles were put on carbon-coated grids, dried at room temperature, examined with a 200-keV field emission transmission-electron microscope (Philips CM 100; Koninklijke Philips Electronics, Eindhoven,

The Netherlands) and analysed by transmission-electron diffraction to identify the TiO_2 crystal phase.

Bulk-TiO_2 and 15-nm TiO_2 nanoparticles were dispersed in PM before treating the cell cultures. Bath sonication for 30 min was used to disperse particle agglomerates in stock solutions.

The suspensions of nanoparticles (1000 μg/ml) were inspected by dynamic light scattering (DLS) using a 3D DLS-SLS (dynamic light scattering – static light scattering spectrometer: LS Instruments, Fribourg, Switzerland). This allows the assessment of hydrodynamic radii of particles in extremely turbid suspensions by a so-called 3D cross-correlation technique that eliminates multiple scattering of light. As the light source, a HeNe laser operating at a wavelength of 632.8 nm was used and scattering was measured at an angle of 90°.

Zeta potentials of TiO_2 nanoparticle suspensions (1000 μg/ml) were measured with ZetaPals, (Brookhaven Instrument Corporation) in the PM medium, and were used to assess the exposure to living cells.

Assessment of cellular fatty acid composition by gas chromatography

T. thermophila cells were harvested by centrifugation at 60 rcf for 10 min of 15 ml culture samples. The pellets were resuspended in sterile double-distilled water (1 ml) then frozen at -20 C and lyophilised. Lipids were transesterified using an HCl/MeOH procedure (Dionisi et al. 1999). Dried samples were pulverised and transferred to screw cap test tubes. First, the sample was mixed with hexane (0.5 ml). Then 1.5M HCl in MeOH (1 ml) and pure MeOH (1 ml) were added and the test tubes were filled with N_2 and incubated at 80 C for 10 min. The reaction was stopped by cooling the tubes in ice. Following the addition of double-distilled water (2 ml), each reaction mixture was vigorously mixed for 1 min and centrifuged for 30 s at 670 rcf. The organic phase was transferred to a vial under N_2 and the samples were stored at -20 C prior to analysis.

Fatty acid methyl esters were separated by capillary gas chromatography using Omegawax TM 320 (30 m × 0.32 mm ID × 0.25 mm) capillary column with polyethylene glycol as the stationary phase. The gas chromatography system used was an Agilent 6890 series GC equipped with Agilent 7683 Automatic Liquid Sampler, 7683 Injector and FID detector and helium as the carrier gas with a flow rate of 2.0 ml/min, split ratio 10:1. The initial temperature for analysis was 185 C and the final temperature was 215°C. The injected volume was 2 μl and the run time was 54 min. Fatty acid methyl esters were identified from their retention times and results were calculated using response factors derived from chromatographic standards of known composition (Nu Chek Prep, GLC-85, Nu-Chek Prep Inc., Elysian, MN, USA).

Results were analysed using ChemStation Plus® software. Membrane fatty acids that were less than 0.5% of total fatty acids were designated as trace fatty acids and were not considered further. Statistical analysis of the compositional data was used to evaluate differences in average fatty acid composition between different treatments (size and concentration of particles). Multivariate analysis of variance on isometric log-ratio transformations of the composition data was carried out.

Assessment of the extent of lipid peroxidation by quantitation of malondialdehyde

Lipid peroxidation was tracked by the formation of malondialdehyde (MDA), a lipid peroxidation by-product that reacts with thiobarbituric acid (Ortega-Villasante et al. 2005). An aliquot of the culture (15 ml) was harvested by centrifugation at 6700 rcf for 10 min. Cells were homogenised by sonication for 3 min in an ice-cold water bath. To measure total protein concentration, 5 μl of sample was taken, and distilled water (995 μl) was added. The sample was then diluted by a factor of 10 and total protein concentration was measured spectrophotometrically at 280 nm. The total protein concentration was used as a measure of the biomaterial in the experiments. For the measurement of MDA concentration, homogenised sample (500 μl) was mixed with buffer A, 30% trichloroacetic acid, 0.75% 2-thiobarbituric acid, 0.5 M HCl and 0.02% butylated hydroxytoluene (500 ml), incubated at 90°C for 30 min, then chilled on ice. n-Butanol (1.5 ml) was mixed with the sample, and the mixture was centrifuged at 6700 rcf for 10 min at 4°C. The absorbance of the resulting chromophore was measured at 535 and 600 nm and the latter was subtracted from the former to correct for nonspecific turbidity. The concentration of MDA was calculated using an extinction coefficient of 156 mM^{-1} cm^{-1} (Ortega-Villasante et al. 2005). For statistical analysis, each concentration of MDA was divided by the total protein concentration of the corresponding sample.

Reactive oxygen species assessment

Assessment of ROS was performed by using the OxiSelect Intracellular ROS Assay Kit™ (Cell Biolabs) measuring green fluorescence as described by Petkovic et al. (2011b). DCFH-DA (2′,7′-dichlorodihydrofluorescin diacetate), standards, H_2O_2 and TiO_2 suspension were prepared in cell media (PM). *T. thermophila* cells were first pretreated with 100 μM solution of DCHF-DA in the PM cell culture media for 60 min at 30 C. Cells were then treated with 250 μM H_2O_2 and 0.1 and 100 μg/ml nano-TiO_2 particles or 0.1 and 100 μg/ml bulk-TiO_2 particles for 4 h. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. H_2O_2 is the principal ROS, responsible for the oxidation of DCFH-DA to DCF (LeBel et al. 1992). Negative (nontreated cells) and positive (H_2O_2-treated cells) controls were included in each experiment. For kinetic analysis of ROS formation, the plates were maintained at 25°C and the fluorescence intensity (480 nm excitation/530 nm emission wavelengths) of the DCF formed was recorded every 5 min (for the first 30 min) and then every 30 min during the remainder of the 4-h incubation, using a Synergy H4 hybrid fluorescence plate reader (BioTrek). The statistical significance between treated groups and controls was determined by two-tailed Student's t-test and $p < 0.05$ was considered as statistically significant. For each concentration of nano- or bulk-TiO_2, three independent assays and two technical replicates were carried out.

Comet assay

Different protocols and versions of Comet assay were used to assess the extent and the type of DNA damage as shown in Figure 1.

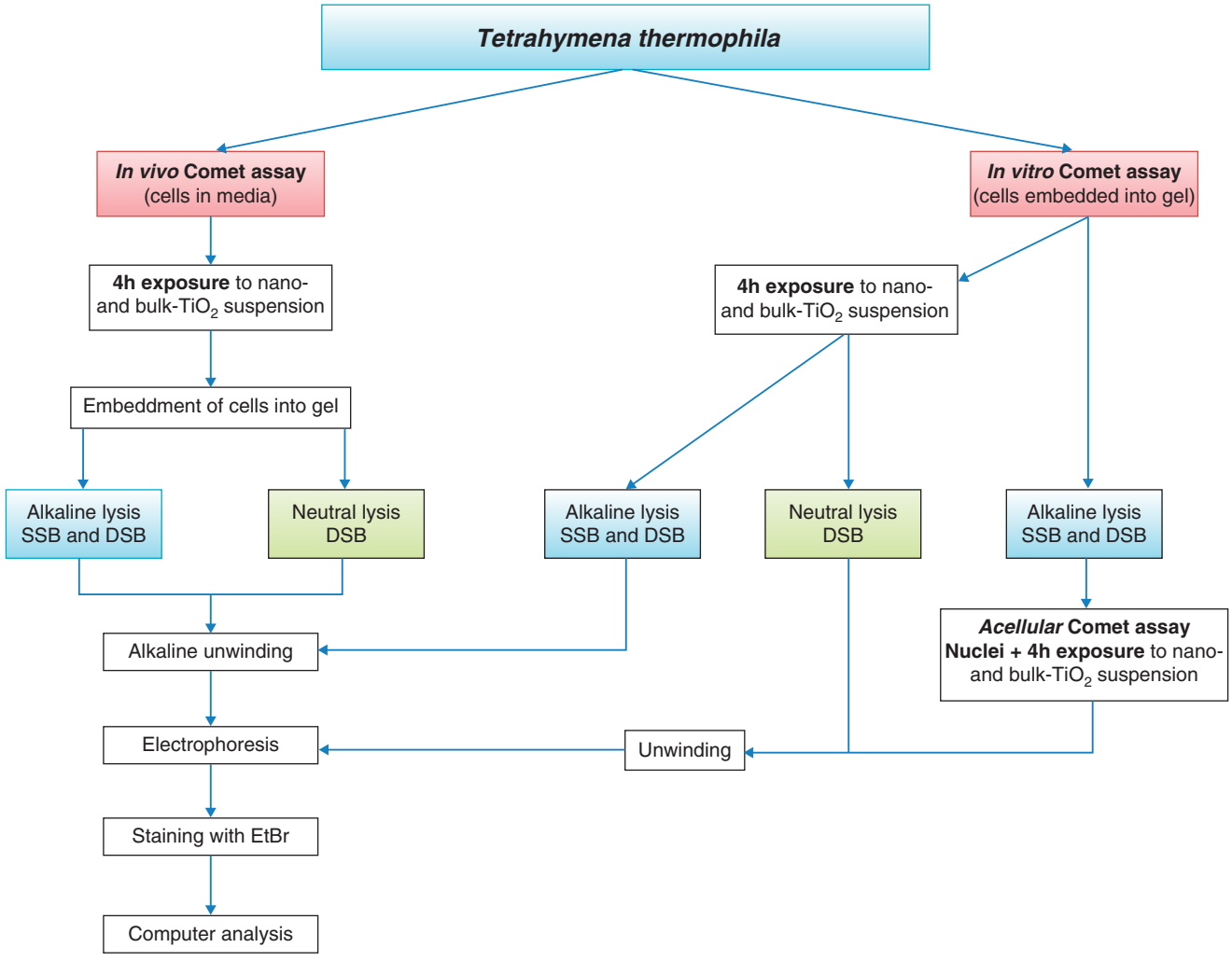

Figure 1. The protocols and types of exposure used in our genotoxicity study. These indicate where and when the bulk- and nano-TiO_2 particles may remain in close proximity to nuclei in the final steps of the Comet assay, leading to an overestimate of genotoxicity and type of DNA damage (DSB, double DNA strand breaks, SSB, single DNA strand breaks).

Comet assay with alkaline lysis *in vivo*

The alkaline version of the Comet assay was performed by modifications of the original protocol (Lah et al. 2004). After exposure to TiO_2 particles in PM (as described in "Exposure Conditions, above), cells were harvested by 5-min centrifugation at 60 rcf washed with PM and resuspended in PM. To achieve a uniform background, rough microscope slides were coated with 400 µl of 0.5% normal melting point (NMP) agarose and were left to air-dry overnight. Cells were mixed with 3.0% low melting point (LMP) agarose and spread over the slides as the second layer, giving a final concentration of 140 cells/µl. After removing the cover glasses, the slides were covered with a third layer of 300 µl of 3.5% LMP agarose, to prevent escape of *T. thermophila* DNA during cell lysis and electrophoresis.

T. thermophila cells embedded in agarose were dipped in phosphate-saline buffer (PBS; 80 g NaCl, 8 g NaCl, 2 g KCl, 2 g KH_2PO_4 in 1 L doubly distilled H_2O at pH 7.2–7.4) for 20 min on ice and then washed twice with PBS. Slides were incubated overnight in lysis solution (30 mM NaOH, 1.2M NaCl, 1% (w/v) lauroylsarcosine, 0.05% Triton X 100, 1% DMSO pH 12.4). The slides were rinsed three times for 20 min each in electrophoresis buffer (30 mM NaOH, 10 mM EDTA, pH 12.4) to remove lysis solution and to unwind the nuclear DNA. The samples were then subjected to electrophoresis for 20 min at 25 V and 300 mA in the same buffer. Following the electrophoresis, the gels were neutralised in 400 mM Tris-HCl, pH 7.5 for 15 min. For visualisation in a fluorescence microscope, the slides were stained with ethidium bromide (10 µg/ml) and 60 randomly selected nuclear images of each slide were acquired with an epifluorescent microscope (Olympus BX50), using a BP 515–560 nm excitation filter and a barrier filter of LP 590 nm at 400× magnification (Figure 2). Microscopic images of comets were captured by a digital camera (Hamamatsu Orca 2), connected to a computer. Detected comets were scored by Komet 5.0 Computer Software (Kinetic Imaging Ltd., 2001). The tail lengths and percentage of DNA in the comet's tails and heads were determined and further used to analyse the nuclear DNA damage.

Comet assay with alkaline lysis *in vitro*

After culture growth in RM for 24 h in the dark at 25°C, cells were harvested by 5 min centrifugation at 60 rcf, washed with

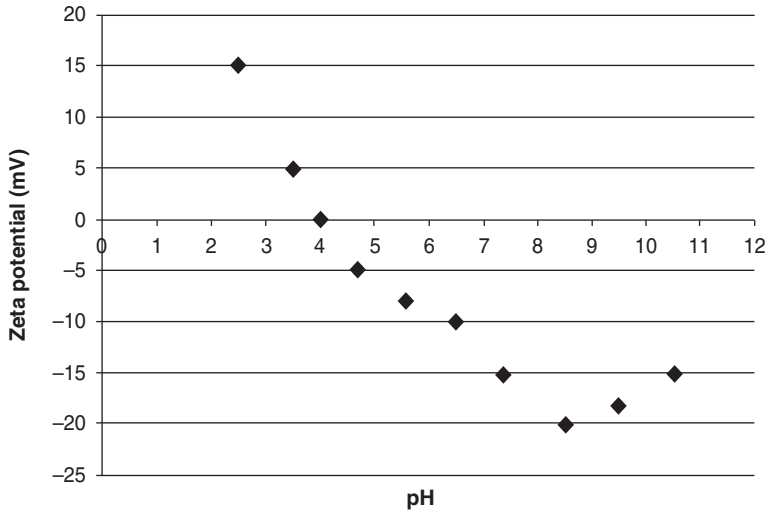

Figure 2. Zeta potentials of TiO_2 nanoparticle suspensions (1000 μg/ml) measured in the poor medium and used in experimental exposures.

PM and resuspended in PM. The cells were embedded into 3.0% low-melting point agarose, the first and the third layers prepared as described above. Glass slides with embedded cells were then exposed to TiO_2 particles in PM for 1 h and then treated with TiO_2 nano and bulk particles (0.1 and 100 μg/ml) for 4 h. *T. thermophila* cells embedded in agarose were dipped in PBS for 20 min on ice and then washed twice with PBS. Glass slides were treated in alkaline lysis and all further steps were the same as described in the section "Comet assay with alkaline lysis *in vivo*" above.

Acellular Comet assay with alkaline lysis

After culture growth in RM for 24 h in the dark at 25°C, cells were harvested by 5 min centrifugation at 60 rcf, then washed with PM and resuspended in PM. The cells were embedded into 3.0% LMP agarose and the first and the third layers were prepared as described above. *T. thermophila* cells embedded on glass slides were dipped in PBS for 20 min on ice and then washed twice with PBS. Glass slides were treated after alkaline lysis and washed three times with PBS buffer for 10 min. One-hour exposure of embedded nuclei to PM in the dark at 25°C was followed by the exposure to TiO_2 particles at two selected concentrations (TiO_2 nano- and bulk particles; 0.1 and 100 μg/ml) for 4 h, in the dark at 25°C. After exposure, the glass slides were washed with electrophoresis buffer (EF buffer; 6 mL NaOH, 4 mL EDTA, 1990 ml MQ), and all further steps were the same as in section "Comet assay with alkaline lysis *in vivo*" above.

Comet assay with neutral lysis *in vivo* and *in vitro*

Both *in vivo* and *in vitro* exposures to TiO_2 particles were tested as described in the sections "Comet assay with alkaline lysis *in vivo*" and "Comet assay with alkaline lysis *in vitro*" above. As a positive genotoxic control toxicant, 100 μM methyl methanesulfonate (MMS) was used. For the Neutral Comet assay, a modification of the protocol by Wojewodzka et al. (2002) was used. The cell suspension was mixed with low-melting point agarose (LMP agarose) at a final concentration of 0.75%. After the preparation of the third layer, the slides were left at 4°C in the dark for 1–2 h in the lysing buffer, which consisted of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 1% *N*-lauroylsarcosine, pH 9.0. Immediately before use, 0.5% Triton X-100 and 10% dimethylsulfoxide (DMSO) were added to the buffer and mixed for 20 min. After 1 h of lysis, the slides were washed three times with the electrophoresis buffer (300 mM sodium acetate, 100 mM Tris–HCl, pH 8.3) and left in fresh buffer solution for 1 h, then placed in a horizontal gel electrophoresis unit filled with a fresh electrophoretic buffer. The slides were electrophoresed for 1 h at 14 V (0.5 V/cm, 11–12 mA) at 8°C.

Statistical analysis of Comet assay results

The average percentage of tail DNA was compared in an incomplete four-factor experimental design using analysis of variance (ANOVA). The first factor was "lysis" with two levels: alkaline and neutral; the second factor was "method" with four levels: *acellular* Comet assay (only by alkaline lysis), *in vivo* Comet assay, *in vitro* Comet assay and control. The third factor was the "size" of TiO_2 particles, either nano or bulk, and the fourth factor was concentration of TiO_2 particles, either 0.1 or 100 μg/ml. The experiment was carried out in three biological replicates. At least 60 nuclei were examined in each replicate and the medians of percentage of tail DNA were calculated for each biological replication. The ANOVA calculations were made on the basis of the medians of percentage of tail DNA. The Duncan's multiple comparison test was used to determine the statistical significant differences between the treatments ($\alpha = 0.05$).

Results

Characterisation of TiO_2 nanoparticle suspensions

The TEM revealed that TiO_2 nanoparticles were homogeneous in shape and size, with an aspect ratio of up to 1:5 between the diameter and length, forming elongated, spheroidal shapes. The transmission-electron diffraction pattern showed the TiO_2 to be in its anatase phase. BET analyses revealed the surface area to be between 190 and 290 m^2/g and the average particle size to be 15 nm.

Table I. Average percentage composition of membrane fatty acid samples from *T. thermophila* exposed to TiO_2 particles at 25° C after 4 h. The data are presented as total sums of various fatty acids of lipid extracted from the three independent cultures. Percentages are expressed as means ± standard error (SE). B, bulk concentration; NP, nanoparticle concentration; FA, fatty acid.

Particle type	Bulk-TiO_2 (B)						Nano-TiO_2 (NP)					
Particle concentration (μg/ml)	0	0.1	1	10	100	1000	0	0.1	1	10	100	1000
Straight chain saturated FA (%)	37.2 ± 0.9	36.2 ± 0.4	37.1 ± 0.8	38.3 ± 0.1	36.0 ± 0.6	37.4 ± 0.4	31.0 ± 0.4	31.0 ± 0.5	30.8 ± 0.8	31.3 ± 0.1	31.5 ± 0.5	32.4 ± 0.2
Unsaturated FA (%)	49.6 ± 1.0	50.1 ± 0.4	50.0 ± 1.1	48.3 ± 0.3	50.6 ± 0.8	49.4 ± 0.4	52.0 ± 0.4	51.6 ± 0.6	52.5 ± 0.6	51.7 ± 0.6	51.5 ± 0.7	50.1 ± 0.4
Monounsaturated FA (1×) (%)	22.4 ± 0.6	21.3 ± 1.3	21.2 ± 0.8	22.6 ± 1.5	21.4 ± 0.5	23.7 ± 1.2	22.3 ± 0.5	22.3 ± 0.5	21.7 ± 1.0	22.7 ± 0.3	21.8 ± 0.6	22.1 ± 0.3
Polyunsaturated FA (2×) (%)	8.3 ± 0.2	8.6 ± 0.2	8.5 ± 0.2	8.0 ± 0.1	8.6 ± 0.2	8.1 ± 0.1	8.8 ± 0.0	8.3 ± 0.5	8.7 ± 0.1	8.7 ± 0.2	8.7 ± 0.1	8.4 ± 0.1
Polyunsaturated FA (3×) (%)	18.9 ± 1.4	20.3 ± 1.3	20.2 ± 1.7	17.7 ± 1.2	20.6 ± 1.1	17.7 ± 1.3	21.0 ± 0.9	21.1 ± 0.3	22.0 ± 1.0	20.3 ± 0.7	21.1 ± 0.4	19.6 ± 0.6
Saturated iso and anteiso branched FA (%)	5.6 ± 0.3	5.4 ± 0.1	5.6 ± 0.2	5.9 ± 0.1	5.4 ± 0.1	5.8 ± 0.2	7.3 ± 0.4	7.5 ± 0.1	7.6 ± 0.1	7.7 ± 0.2	7.5 ± 0.2	7.8 ± 0.3
Saturated iso FA (%)	5.0 ± 0.3	4.8 ± 0.1	5.0 ± 0.2	5.2 ± 0.1	4.8 ± 0.1	5.1 ± 0.1	6.5 ± 0.1	6.4 ± 0.1	6.5 ± 0.0	6.6 ± 0.2	6.5 ± 0.1	6.7 ± 0.3
Saturated anteiso FA (%)	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.4	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
Average number of C-atoms in membrane FA	14.7	14.9	15.0	14.9	14.9	15.0	14.7	14.7	14.9	15.0	14.7	14.6

Dynamic light scattering analysis showed the average value of the hydrodynamic radius *R*h, of TiO_2 nanoparticles suspended in test medium to be 820 nm. The average size of bulk-TiO_2 could not be measured accurately with this approach because of the presence of larger agglomerates.

Zeta potentials of TiO_2 nanoparticle suspension (1000 μg/ml) were measured in the same medium used to expose cells, at pH 7.4. The value recorded was -15, which is equivalent to a suspension of incipient stability (Figure 2).

Cellular fatty ACID composition

No significant differences have been found in membrane fatty acid profiles of *T. thermophila* after exposure to different concentrations of nano- or bulk-TiO_2 at 25°C after 4 h (Table I). This suggests that TiO_2 particles have no effect on *T. thermophila* cell membranes.

Lipid peroxidation

There were no differences in lipid peroxidation of analysed *T. thermophila* samples after 0 and 4 h of incubation at 25°C with nanoparticles, when compared with control cells. The average content of malondialdehyde in the control samples was 140 ± 23 nM of MDA per milligram of protein.

Reactive oxygen species production

In comparison with control cells, no ROS production was detected after 4 h of incubation with TiO_2 particles of any size at a concentration of 0.1 μg/ml; however, at 100 μg/ml bulk-TiO_2, but not nano-TiO_2, significant elevation of intracellular ROS formation was detected (Figure 3A).

To explore whether TiO_2 nanoparticles (0.1 and 100 μg/ml) induced ROS formation not only at the end of exposure but also during the experiment, we measured the kinetics of their formation in *T. thermophila* cells in different time frames during 4 h of exposure (Figure 3B). Comparison between treated groups and controls tested by two-tailed Student's t-test and $p < 0.05$ showed no statistically significant changes in ROS formation.

Comet assay

Statistical analysis of the results obtained with a Comet assay after alkaline lysis indicated significant damage of DNA in *T. thermophila* in both *in vivo* and *in vitro* treatments with TiO_2 in comparison with control. This was independent of both the size and the concentration of particles (Figure 4).

Statistical analysis of results of a Comet assay obtained with embedded nuclei (*acellular* exposure) also showed significant DNA damage at all TiO_2 exposure concentrations and sizes used, except for 100 μg/ml nano-TiO_2 concentration. Statistically significant differences, calculated using Duncan's multiple comparison test, between DNA damage in the two exposure concentrations of particles have been observed in *acellular* Comet assays. A possible explanation for this is that nano- and bulk particles in suspensions aggregate more at higher concentrations (100 μg/ml) and this may hinder penetration into the gels.

Statistical analysis of Comet assays by neutral lysis showed that in cells treated with TiO_2, the average DNA tail length does not significantly differ from that in control cells,

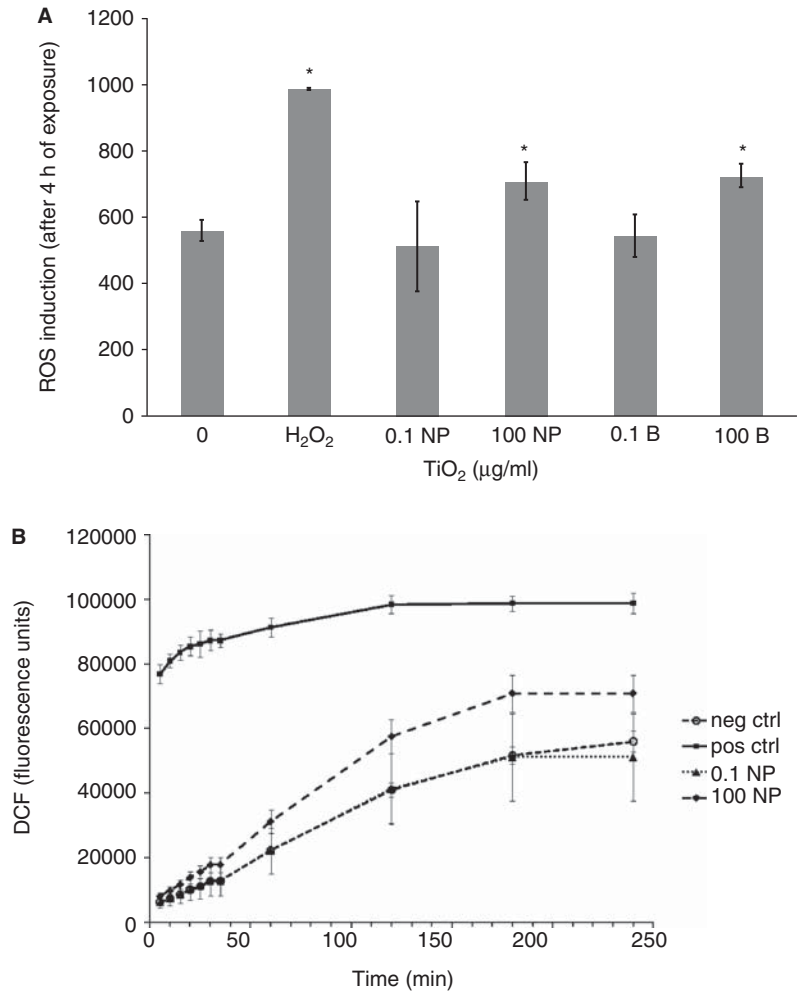

Figure 3. Induction of reactive oxygen species (ROS) formation in *T. thermophila* cells. (A) treated with H_2O_2 (250 μM= pos ctrl), nano-TiO_2 particles (NP, 0.0 μg TiO2/ml = neg ctrl, 0.1 and 100 μg/ml), bulk-TiO_2 particles (0.1 and 100 μg/ml) and presented as a relative increase in DCF fluorescence after 4 h of exposure to TiO_2 particles. Each bar is represented as a mean ± standard error (SE) of three independent experiments. (B) Kinetics of ROS formation during exposure for 4 h to TiO_2 NPs (0.1 and 100 μg/ml). Each point represents the mean of six replicates ± standard error (SE).

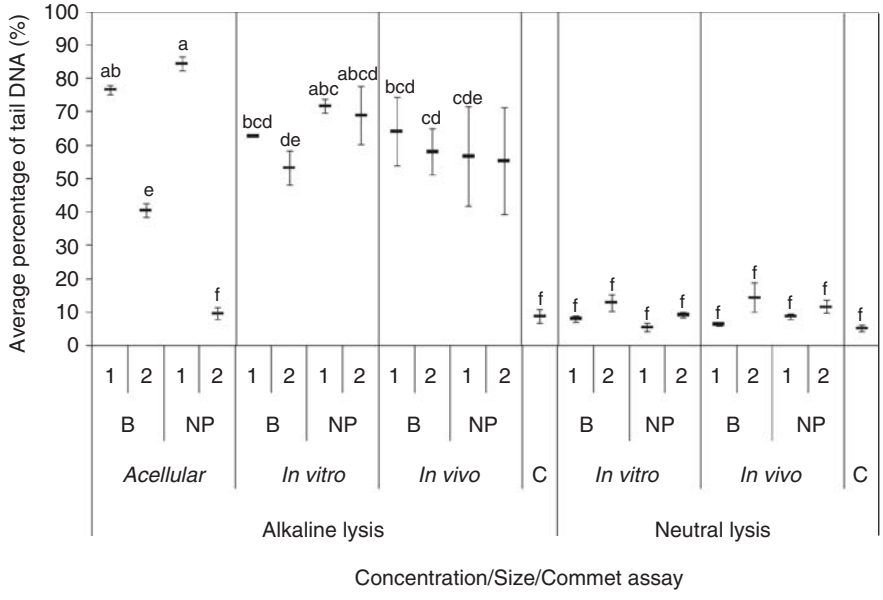

Figure 4. Results of the Comet assay experiment are presented as an average percentage of tail DNA (assessed in 60 cells). *T. thermophila* was treated with nanoparticles (NP) or bulk TiO_2 particles (B) at two different concentrations (1 - 0.1 μg/ml and 2 - 100 μg/ml). Control group was not treated with TiO_2 and is indicated as "C". Three different exposure scenarios (acellular, *in vitro*, *in vivo*) were applied and two different protocols of Comet assay (alkaline lysis and neutral lysis). There is no statistically significant difference between averages indicated with the same letter (a,b,..., f).

indicating no double-strand breaks occur as a result of exposure to TiO_2 (Figure 4). Double strand breaks did not occur in bulk- or in nano-TiO_2 treated cells. Concentration and exposure type, namely in *in vivo* or *in vitro* experiments, failed to produce double strand breaks in DNA. When cells were treated with 100 μM MMS, a reference positive control for double-strand breaks, a statistically significant level of DNA damage was recorded.

Simultaneous performance of alkaline lysis and neutral lysis in this study indicates that single strand breaks are the main category of DNA damage caused by TiO_2 particles. No double-strand breaks were observed (Figure 4). The results of the *acellular* exposure to TiO_2 revealed the capacity of TiO_2 particles to produce extensive single-strand breaks when interacting with embedded nuclei and imply that when TiO_2 particles are present in the medium during in a Comet assay, they can interfere with DNA and give rise to false-positive results and overestimates of actual genotoxicity.

Discussion

We report experimental evidence of TiO_2 particle interactions with DNA during the Comet assay that resulted in a positive test result. We studied the DNA damage sustained by *T. thermophila* incubated with TiO_2 bulk- and nanoparticles and assessed by a Comet assay, and we analysed cellular responses, including lipid peroxidation, ROS formation and membrane fatty acid profiles. The DNA was exposed to particles in three different exposure scenarios in order to assess whether nanoparticles could directly interact with DNA during the course of the assay – and thus produce a false-positive result or an overestimate of the actual genotoxicity.

The ability of TiO_2-NPs to damage DNA has been shown in many studies (Gurr et al. 2005; Wang et al. 1998; Trouiller et al. 2009; see (Karlsson 2010); see (Sathya et al. 2010)), but it has generally been rationalised as a consequence of oxidative stress. Our results showed that only TiO_2 bulk particles at 100 μg/ml cause significant ROS production, a result never observed with nanoparticles. Other cellular markers such as membrane fatty acid profiles and lipid peroxidation, which could be also regarded as markers of cytotoxicity, remained unchanged compared with control cells. ROS elevation by bulk-TiO_2 particles (100 μg/ml) does not imply a higher degree of DNA damage and these results clearly indicate that oxidative stress is not a cause of the genotoxicity, which was detected in our Comet assay study. Consequently, the recorded genotoxicity must be either independent of oxidative stress or a false-positive result. Since literature data failed to report direct primary genotoxicity but rather genotoxicity driven by oxidative stress, the Comet assay results would appear to be false positives. There is only one published study in which, judging by an alkaline Comet assay, no genotoxicity was observed with nanoparticulate TiO_2 (Bhattacharya et al. 2009). We hypothesise that in this case, complete removal of the nanoparticles from the test system was achieved and the particles were not endocytosed to any significant degree. Potential causes of false-positive results include particles that may remain in the test medium or particles that are present in food vacuoles or have been endocytosed. That intracellular particles can gain access to DNA after lysis in the course of a Comet assay has been discussed by Stone et al. (2009) and by Karlsson (2010).

Our results agree with those in other reports. A review by Landsiedel et al. (2009) reported results of nanomaterial genotoxicity tests, which were dependent on the tests themselves. In an assessment of the genotoxicity of nanoparticles, in six studies, the Ames test showed no genotoxicity, and this was associated with a barrier to penetration by the nanomaterials through the bacterial cell wall. By contrast, of 14 *in vitro* micronucleus assays, 12 produced evidence of genotoxicity, and in the Comet assay, 14 of 19 studies showed nanomaterials to be highly genotoxic (Landsiedel et al. 2009). A partial explanation for these inconsistencies among the tests may be the fact that the Comet assay is the most sensitive of the assays, but since different concentrations of nanoparticles were applied in the studies, this suggestion cannot be a complete explanation and other factors, such as direct interaction of NPs with DNA during the tests, should be considered.

Based on the results of our study presented here, we suggest that when the Comet assay is selected for assessment of genotoxicity of nanoparticles, pretesting of potential of nanoparticles to interact with DNA *post festum* must be carried out. One means by which such interactions could be detected is use of the *acellular* Comet test. In addition, before settling on the Comet assay, it is important to know whether to expect substantial amounts of intracellular nanoparticles that could interact with DNA while the test is proceeding.

Suspected genotoxicity should be confirmed by an independent assay or, at a minimum, with biomarkers indicating DNA repair, for example, mRNA expression of tumour suppressor gene p53 and its downstream regulated responsive genes (Petkovic et al. 2011b), DNA deletions (Trouiller et al. 2009), inflammation (Trouiller et al. 2009, Grassian et al. 2007), or indications of oxidative stress status such as lipid peroxidation, or elevated levels of ROS (Gurr et al. 2005; Kang et al. 2008).

In isolation, the results of Comet assays are unreliable as a measure of nanoparticles' genotoxicity due to the possibility of false positives. In the future, the test protocol needs modifications in terms of exclusion or control of particle–assay interactions and combination with other oxidative stress markers. Only with such refinements will the Comet assay remain a test capable of reliably confirming or disproving genotoxicity.

Conclusions

(1) Genotoxicity of TiO_2 nanoparticles was demonstrated when *T. thermophila* cells were incubated with nano-TiO_2 or bulk-TiO_2 in a suspension (*in vivo* exposure), or embedded in gels (*in vitro* exposure) or when only embedded nuclei (*acellular* exposure) were exposed to nanoparticles (Figure 1). Since positive Comet assay results were not accompanied by cytotoxicity markers such as lipid peroxidation, ROS formation or changes in composition of cell membranes, our Comet assay results appear to represent a false positive.

(2) We suggest that in the future, pretesting of particle DNA interactions should be conducted in an *acellular* Comet assay and only the Comet assay results consistent with this pretesting should be accepted.
(3) Data obtained from a Comet assay method alone are inadequate to support an assertion of an enhancement of the genotoxic potential of NPs. The genotoxic potential of NPs as obtained by a Comet assay should be accepted only when combined with evidence adduced by properly selected oxidative stress biomarkers.

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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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