Toxicology Letters 215 (2012) 54–61

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Modifications of the bacterial reverse mutation test reveals mutagenicity of TiO_2 nanoparticles and byproducts from a sunscreen TiO_2-based nanocomposite

Stéphane Jomini[a], Jérôme Labille[b,c], Pascale Bauda[a,c], Christophe Pagnout[a,c,*]

[a] Laboratoire des Interactions Ecotoxicologie, Biodiversité, Ecosystèmes (LIEBE), UMR 7146, CNRS-UPV-M, Université de Lorraine, rue du Général Delestraint, F-57070 Metz, France
[b] CEREGE UMR 6635 CNRS/Aix-Marseille Université, Europôle de l'Arbois, 13545 Aix-en-Provence, France
[c] International Consortium for the Environmental Implications of Nanotechnology (iCEINT), Europole de l'Arbois, F-13545 Aix en Provence, France

HIGHLIGHTS

- The Ames test is not suitable for nanoparticle (NP) genotoxicity assessment.
- The Ames test medium prevents electrostatic interactions between bacteria and NPs.
- The Ames test medium strongly promotes the aggregation of NPs.
- Simple pre-exposure step in an adequate medium improve the accuracy of the test.
- Modified Ames test showed mutagenicity of NP-TiO_2 and NP-TiO_2-based nanocomposite.

ARTICLE INFO

Article history:
Received 30 May 2012
Received in revised form 8 August 2012
Accepted 19 September 2012
Available online 28 September 2012

Keywords:
TiO_2 nanoparticles
Genotoxicity
Bacterial reverse mutation test
Fluctuation test
Electrostatic interactions

ABSTRACT

The bacterial reverse mutation test, recommended by the Organization for Economic Co-operation and Development (OECD) to determine genotoxicity of chemical compounds, has been recently used by several authors to investigate nanoparticles. Surprisingly, test results have been negative, whereas in vitro mammalian cell tests often give positive genotoxic responses. In the present study, we used the fluctuation test procedure with the *Salmonella typhimurium* strains TA97a, TA98, TA100 and TA102 to determine the mutagenic potential of TiO_2 nanoparticles (NP-TiO_2) and showed that, when it is used conventionally, this test is not suitable for nanoparticle genotoxicity assessment. Indeed, the medium used during exposure prevents electrostatic interactions between bacterial cells and nanoparticles, leading to false-negative responses. We showed that a simple pre-exposure of bacteria to NP-TiO_2 in a low ionic strength solution (NaCl 10 mM) at a pH below the nanoparticle isoelectric points (pH 5.5) can strongly improve the accuracy of the test. Thus, based on these improvements, we have demonstrated the genotoxicity of the engineered NP-TiO_2 tested and a NP-TiO_2 byproduct from a sunscreen nanocomposite. It was also shown that strain TA102 is more sensitive than the other strains, suggesting an oxidative stress-mediated mechanism of genotoxicity.

1. Introduction

Due to the increasing industrialization of many countries and resulting technological advances, environmental pollution has become a serious health issue. Many efforts have been made to protect the environment and human health. A priority area concerns the development of in vitro assays to evaluate the toxicological effects of environmental chemicals and then build prioritization models of in vivo toxicity. Over the years, multiple bioassays have been developed utilizing many organisms. Microbial tests have several advantages over other bioassays, including rapid response times due to the much shorter microbial life cycles, reproducibility of test conditions, amenability to genetic manipulations, increased sensitivity and reduced cost (Davoren, 2005). In addition, microorganisms possess the majority of the same biochemical pathways present in higher organisms, they exhibit significant membrane structure organization and generally elicit toxic responses to many chemicals through mechanisms similar to that of higher organisms (Qureshi et al., 1984).

The bacterial reverse mutation assay is recommended by national and international environmental protection agencies for substance evaluations (e.g. Organization for Economic Co-operation and Development (OECD test guideline 471); and The International Conference on Harmonization (ICH)) (Mortelmans

* Corresponding author at: Laboratoire des Interactions Ecotoxicologie, Biodiversité, Ecosystèmes (LIEBE), CNRS-UMR 7146, Université de Lorraine, rue du Général Delestraint, F-57070 Metz, France. Tel.: +33 387378657; fax: +33 387378512.
E-mail address: christophe.pagnout@univ-lorraine.fr (C. Pagnout).

0378-4274/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2012.09.012

and Zeiger, 2000). This test has also been recently approved as one of the two assays recommended by The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) (Kirkland et al., 2011). The bacterial reverse mutation test uses several strains of *Salmonella typhimurium* with mutated histidine synthesis genes. The test principle is based on the fact that reverse mutations caused by exposure to mutagenic compounds can reactivate the ability of mutated bacterial strains to synthesis histidine, thereby allowing them to grow in the absence of this essential amino acid. Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method, commonly named the Ames test (Ames et al., 1972, 1973a,b), the preincubation method (Maron and Ames, 1983; Aeschbacher et al., 1987), the fluctuation method (Green et al., 1976; Hubbard et al., 1984; McPherson and Nestmann, 1990), and the suspension method (Thompson and Melampy, 1981). Over the years, many validation studies have been performed to determine the sensitivity and correlation of this test with animal carcinogenicity studies. It has been established that there is a high predictivity of a positive mutagenic response in the test for rodent carcinogenicity ranging from 77% to 90% (McCann et al., 1975; Tennant et al., 1987; Zeiger, 1998). To date, there are thousands of research and testing laboratories throughout the world using this assay to screen potentially mutagenic drugs and chemicals. Many companies and regulatory agencies use the results from this assay as part of their short-term toxicological testing programs to determine chemical safety (Felton and Wu, 2003).

Nanotechnology is a relatively new branch of science, heralded as a technological revolution (The White House, 2000). Engineered nanoparticles have rapidly moved from the laboratory to industry and are currently being used in many consumer products. Currently, there are over 1000 products in the consumer marketplace that include nanomaterials (Woodrow Wilson Database: http://www.nanotechproject.org), which is projected to substantially increase in the near future. This phenomenon has aroused great concern about potential human health effects and, on a larger scale, environmental effects (Nel et al., 2006), giving birth to a new biological field known as "Nanotoxicology". The Royal Society and Royal Academy of Engineering first raised this concern in 2003 (The Royal Society and the Royal Academy of Engineering, 2003; The Royal Society, 2004), paving the way for a rapid increase in investigational studies on nanoparticle toxicity; in particular, genotoxicity studies, as many nanoparticles were found to cause chromosomal aberrations, DNA strand breaks, oxidative DNA damage, and subsequent genetic mutations (Singh et al., 2009). In commonly used in vitro (chromosomal aberrations, comet assay, micronucleus) and in vivo mammalian test cell systems, nanoparticles have been largely found to promote positive genotoxic responses, while negative responses have been generally obtained for these nanoparticles with the bacterial reverse mutation test (Doak et al., 2012). It was reported that within 19 published studies, where this test was used for the genotoxicological analysis of nanoparticles, 17 showed negative mutagenicity. The two remaining studies only revealed weak mutagenic effects. Therefore, these studies seemed to have indicated that although the Ames test is excellent for testing chemical mutagenic activity, it does not appear to be suitable for nanoparticles. This might be related to the degree of nanoparticle uptake by bacterial cells, which is likely to be less than in mammalian cells (Singh et al., 2009; Doak et al., 2012). Indeed, bacteria cannot perform endocytosis and their cell wall forms a barrier against simple diffusion of nanoparticles. This lack of uptake could potentially lead to false negative results.

Based on our previous work (Pagnout et al., 2012), we think that another plausible hypothesis that leads to false negative results is the lack of interactions between nanoparticles and bacterial cells due to the use of an inappropriate medium during the exposure. We also think that performing the bacterial reverse mutation test by the fluctuation method instead of the plate incorporation method, with a pre-exposure step in a low-ionic strength solution at a pH value below the nanoparticle isoelectric points (NaCl 10 mM, pH 5.5), could improve these interactions and make the test more accurate for the assessment of the nanoparticle genotoxicity. As a consequence, in the present study, we assessed the mutagenic potential of two engineered TiO_2 nanoparticles and a TiO_2-byproduct derived from a nanocomposite material commonly used in sunscreens with the conventional fluctuation test and with a modified version of this test according to the modification mentioned above. TiO_2 nanoparticles (NP-TiO_2) were used as a model in this study for the following reasons: (i) these nanoparticles are widely used in consumer products (e.g. paints, plastics, paper, ceramics, cosmetics, and sunscreens) with expanded applications over the last decade (Colvin, 2003; Gleiche et al., 2006); (ii) in 2006, TiO_2 was reclassified from *Unclassifiable as to carcinogenicity in humans* (Group 3 carcinogen) to *Possibly carcinogenic to humans* (Group 2B carcinogen) based on sufficient evidence using experimental animals (Ng et al., 2010); (iii) NP-TiO_2 was recently listed by the OECD as one of the priority nanomaterials for immediate testing (OECD, 2008); (iv) NP-TiO_2 nanoparticles are minimally water-soluble and their potential carcinogenic effects cannot be attributed to the release of titanium ions in the medium; and (v) several studies showed no mutagenicity (Warheit et al., 2007; Pan et al., 2010) or very weak mutagenicity (Kumar et al., 2011) caused by NP-TiO_2 with the bacterial reverse mutation test (plate incorporation procedure), whereas NP-TiO_2 has been found to have positive genotoxic responses in other in vitro cellular test systems (Balasubramanyam et al., 2009; Di Virgilio et al., 2010; Osman et al., 2010; Shi et al., 2010).

2. Materials and methods

2.1. Evaluated nanomaterials

TiO_2 nanopowder AEROXIDE® P25 (TiO_2-P25) was provided by Evonik Degussa GmbH (Frankfurt, Germany, Stock # 4168050298). These nanoparticles are described by the supplier as having a primary size of 25 nm with a specific surface area (SSA) of 50 ± 15 m^2/g and a ratio of anatase/rutile forms of 80/20. The TiO_2-P25 stock suspension was prepared by dispersing 100 mg of NP-TiO_2 in 10 mL of sterile ultrapure water (milli-Q water, 18.2 MΩ cm). The resultant suspension was then probe-sonicated (Sonics Vibra-cell 750 W, Sonics & Materials, Inc., Newton, CT, USA; frequency 20 kHz, 3 mm micro tip, amplitude 40%) for 30 min at 4 °C to homogenize and break the larger agglomerates apart (Pagnout et al., 2012).

The second type of NP-TiO_2 used in this study (TiO_2-NA) was provided as a 15% (w/v) stable suspension in acidified water produced by Nanostructured & Amorphous Materials, Inc. (Houston, TX, USA – Stock # 7012WJWR). These nanoparticles are described by the supplier as being 100% anatase, with a primary size ranging from 5 to 30 nm, a SSA of 200–220 m^2/g, and a purity >99.5%. The stock suspension was prepared to 10 g/L by dilution in sterile ultrapure water.

The third nanomaterial used in this study was a byproduct obtained after alteration of a TiO_2-based nanocomposite, namely T-Lite™ SF (BASF, Germany) (TiO_2-TLB), which is commonly used in sunscreens as a UV blocker. The T-Lite nanocomposite consisted of a TiO_2 rutile core (5–10 nm cross-section per 50–200 nm length) arranged together in large clusters, which had an average size of 200 nm. These clusters were embedded in an amorphous layer of aluminum oxide [$Al(OH)_3$] and polydimethylsiloxane (PDMS) (Labille et al., 2010; Auffan et al., 2010). The byproduct, resulting from an accelerated ageing process, was provided by Jérôme Labille (CEREGE Laboratory, Aix-en-Provence, France). Briefly, the alteration process consisted of mixing 100 mg of TiO_2-TLB in 250 mL of ultrapure water. The mixture was magnetically stirred at 690 rpm under a white light (400 W Philips® 114 Master HPI-T Plus) for 48 h. After alteration, this mixture was settled for 48 h and the supernatant containing stable altered TiO_2 nanocomposites (byproducts) was obtained (Labille et al., 2010; Auffan et al., 2010). As previously described by Bigorgne et al. (2011), the quantity of TiO_2 byproducts was measured by filtering an aliquot of suspension (20 mL) through a 25 nm membrane filter and drying filter at 105 °C for 24 h. The concentration of TiO_2 byproducts obtained was adjusted to 100 mg/L with ultrapure water.

2.2. Physicochemical characterization of nanomaterials

The shapes and primary sizes of nanoparticles were determined by transmission electron microscopy (TEM), using a CM 20 Philips electron microscope at 200 kV at the Service Commun de Microscopies Electroniques et de Microanalyses (SCMEM, Nancy, France). Samples were prepared by evaporating a droplet of nanoparticle suspension on a copper grid at room temperature. Size was determined using 100 randomly chosen nanoparticles. Nanoparticle crystalline structures were determined by X-ray powder diffraction (XRD). Electrophoretic mobility and nanoparticle size distribution measurements in aqueous media were performed with a ZetaSizer 3000HS (Malvern Instruments, Worcestershire, UK).

2.3. Bacterial electrophoretic mobility

Bacterial electrophoretic mobility measurements were conducted using a Zetaphoremeter IV (CAD Instrumentations, Les Essarts le Roi, France) in a quartz Suprasil® cell (Heraeus Quarzglas GmbH & Co., Hanau, Germany) at 24 °C from the reflection of a laser beam by bacteria tracked with a charge-coupled device camera. Image analysis software was used to process recorded images in real time to calculate the electrophoretic mobilities from the displacement (migration motion) of bacteria subjected to a constant direct-current electric field (800 V/m). Different cycles were recorded to yield 100 bacterial mobility measurements.

2.4. The conventional fluctuation test

The bacterial reverse mutation test was performed using *S. typhimurium* strains TA97a, TA98 to detect GC frameshift mutations, strain TA100 for GC base-pair substitution mutations and strain TA102 for AT base-pair substitution mutations, without S9 metabolic activation. These strains were a gift from Bruce Ames (University of California, Berkeley, CA, USA). The genetic integrities of these strains were verified before the tests for histidine/biotin, rfa marker and pKM101 plasmid (Maron and Ames, 1983). The test was conducted in liquid medium in 96-well microtiter plates, according to the fluctuation method (Environment Canada, 1993). Briefly, bacterial strains were grown overnight in Oxoid broth supplemented with the appropriate antibiotics (25 μg/mL Ampicillin for strains TA97a, TA98, and TA100 and 25 μg/mL Ampicillin + 2 μg/mL Tetracycline for strain TA102) at 37 °C while shaking. To a volume of 2.5 mL of the Ames reagent mixture (108 mL of 5.5× concentrated Davis–Mingioli salts) [38.5 g/L K_2HPO_4, 11.0 g/L KH_2PO_4, 5.5 g/L $(NH_4)_2SO_4$, 1.375 g/L sodium citrate, 0.55 g/L $MgSO_4 \cdot 7H_2O$ – autoclaved 15 min at 121 °C], 24 mL of 40% (w/v) D-glucose (6 mL of D-biotin [0.1 mg/mL], 0.3 mL of L-histidine [1 mg/mL] and 12 mL of bromocresol purple [2 mg/mL]), 5 μL of an overnight culture and 17.5 mL of sterile ultrapure water containing nanoparticles (1, 10, 100 mg/L or water as a control) were added, giving final exposure NP-TiO_2 concentrations of 0, 0.875, 8.75, 87.5 mg/L. A 200 μL volume of the mixture was dispensed into wells of 96-well microtiter plates and incubated for 5 days at 37 °C in the dark. Classical positive controls, 9-aminoacridine (10 μg/mL), 2-nitrofluoren (0.4 μg/mL), sodium azide (25 ng/mL) and cumene hydroperoxide (3.5 μg/mL), were used for TA97a, TA98, TA100 and TA102, respectively. Distillated water was used as negative control. Sterility controls (i.e. sterility of the media, sterility of the NP-TiO_2 and sterility of the positive controls) were also included for each test. The test was considered valid only if the positive controls induced a number of positive wells significantly higher than the number obtained with the negative control (spontaneous revertants), and only if the wells of all the sterility controls stayed negative during the experimentation (0 positive well on the 96 tested).

2.5. The modified fluctuation test

Bacterial strains were grown overnight in Oxoid broth supplemented with the appropriate antibiotic (25 μg/mL Ampicillin for TA97a, TA98, TA100 and 25 μg/mL Ampicillin + 2 μg/mL Tetracycline for TA102) at 37 °C while shaking. A volume of 2 mL of these overnight cultures were transferred to 500 mL Erlenmeyer flasks containing 200 mL of Luria–Bertani medium with the appropriate antibiotics and incubated at 37 °C while shaking until they reached an optical density (OD) of 0.5–0.6 at 600 nm (exponential growth phase). The bacterial cells were then centrifuged at 8000 × *g* for 10 min at 4 °C, washed twice with NaCl 10 mM (ultrapure water – pH 5.5), and resuspended in the same saline solution at a final OD of 4.0 at 600 nm. A 200 μL volume of these bacterial suspensions was transferred to 100 mL flasks containing 20 mL of NaCl 10 mM (ultrapure water – pH 5.5) and nanoparticles (1 mg/L, 10 mg/L or 100 mg/L or water as a control). After a short pre-exposure (0.1 h), 10 h or 20 h at 20 °C under agitation (150 rpm), 500 μL aliquots were transferred to 2.5 mL of the Ames reagent mixture (previously described) and 17 mL of sterile distilled water, giving final concentrations of NP-TiO_2 in the fluctuation test of 0, 0.025, 0.25 and 2.5 mg/L. A 200 μL volume of the mixture thus obtained was dispensed into 96-well microtiter plates and incubated for 5 days at 37 °C in the dark. Experiments were performed independently twice and one microplate was used for each concentration. When these experiments gave contradictory results, the experiment was repeated a third time. Classical positive, negative, and sterility controls were also included as previously described in Section 2.4.

2.6. Transmission electron microscopy of bacterial cells

Transmission electron microscopy (TEM) observations were realized to study NPs and bacterial interactions. After exposure, bacterial suspension was centrifuged at 10,000 × *g* for 10 min at 4 °C. Then, the cells were fixed with a 2.5% glutaraldehyde solution, washed twice with ultrapure water, post-fixed with osmium tetraoxide (OsO_4), washed three times with ultrapure water, and then, dehydrated in graded concentrations of ethanol (Strum et al., 1971). The pellet obtained was embedded in Epon and polymerized for 2 h at 38 °C and 2 days at 60 °C. Ultra-thin sections (90 nm) were collected over copper grids and counterstained with lead citrate and uranyl acetate. These sections were then observed with a CM 20 Philips electron microscope at 200 kV at the Service Commun de Microscopies Electroniques et de Microanalyses (SCMEM, Nancy, France).

3. Results and discussion

3.1. NP-TiO_2 characterization

The NP-TiO_2 used in this study has been thoroughly characterized. Analyses confirmed that TiO_2-P25 is a mixture of anatase and rutile forms (~84% anatase and 16% rutile) with an average primary particle size of 23 ± 4.9 nm (Supporting Information Fig. S1). Dynamic light scattering (DLS) measurements revealed that the average hydrodynamic diameter of the nanoparticle stock suspension obtained after dispersion in milli-Q water and probe sonication ranged between 60 and 80 nm (Supporting Information Fig. S1), meaning that the nanoparticles were agglomerated. The isoelectric point of TiO_2-P25 was previously determined to be approximately pH 6.8 (Pagnout et al., 2012).

TiO_2-NA, which was reported to be 100% anatase, was found to be a mixture of anatase and brookite forms (~86% anatase and 14% brookite) with an average primary particle size of 5.7 ± 1.9 nm (Supporting Information Fig. S2). DLS measurements revealed that the stock suspension provided by NanoAmor, Inc. was well dispersed with an average hydrodynamic diameter of the nanoparticles between 5 and 10 nm (Supporting Information Fig. S2). The isoelectric point of the TiO_2-NA was found to be around pH 6.5 (data not shown).

TiO_2-TLB, resulting from an accelerated ageing procedure of the TiO_2-based nanocomposite T-Lite™ SF, was provided and fully characterized by Labille et al. (2010) and Auffan et al. (2010) (the same stock suspension was used for the present study). It was shown that the alteration process removed most of the PDMS layer and produced stable suspensions of TiO_2 byproduct with a size ranging from 100 to 700 nm. ICP-AES measurements did not detect any measurable concentration in solution and Al was clearly detected by TEM-EDS analysis on the altered nanocomposite, indicating that the Al remained mainly under a solid form at the nanocomposite surface. The isoelectric point of the TiO_2-TLB was found to be between pH 7 and 8 (Labille et al., 2010).

3.2. Mutagenicity assay using the conventional fluctuation test

The bacterial reverse mutation test was performed using *S. typhimurium* strains TA97a, TA98, TA100 and TA102 as described in Section 2 to evaluate the genotoxic potential of NP-TiO_2. The test was conducted in liquid medium in 96-well microtiter plates, according to the fluctuation method. The fluctuation test was shown to be more sensitive than the plate incorporation method, probably because of the greater diffusion of tested compounds in liquid medium than in solid medium. However, exposition of *S. typhimurium* strains to TiO_2-P25, TiO_2-NA and TiO_2-TLB induced no positive response (Table 1).

These results confirm those obtained by Warheit et al. (2007) and Pan et al. (2010), both showed negative mutagenicity of the TiO_2 nanoparticles with the bacterial reverse mutation test (plate incorporation procedure, or Ames test), even at high concentrations. They also reinforce the observations made by Doak et al.

Table 1
Number of revertants obtained with the conventional fluctuation test.

	Concentration (mg/L)	*Salmonella typhimurium* strains			
		TA97a	TA98	TA100	TA102
TiO_2-P25	0	35/40	11/9	15/12	60/62
	0.875	33/36	10/11	16/14	59/60
	8.75	35/35	10/8	19/15	61/63
	87.5	32/34	9/10	18/16	60/59
TiO_2-NA	0	39/33	13/10	12/15	61/60
	0.875	40/36	11/12	14/13	63/61
	8.75	39/34	12/11	11/13	65/60
	87.5	32/30	10/9	12/11	60/59
TiO_2-TLB	0	30/36	11/14	19/15	59/63
	0.875	33/34	9/13	16/14	58/62
	8.75	34/33	12/11	17/14	61/63
	87.5	33/35	10/12	16/13	59/60
Positive controls					
9-AA	10	96/96			
2-NF	0.4		92/94		
SA	0.025			95/91	
CH	3.5				96/96

Numbers of revertants statically different (Chi-square analysis (Gilbert, 1980)) from the number of spontaneous revertants obtained with the control are underlined. Experiments were performed independently twice and one 96-well microplate was used for each concentration. Mutagenicity of NP-TiO_2 is considered when the tests were positive twice (results highlighted in gray).

(2012) that many nanoparticles are negative for mutagenicity in the Ames test, whereas they have widely been found to have positive genotoxic responses in other in vitro mammalian cell test systems. Of 19 studies published, in which this test was used for genotoxicological analysis of nanoparticles, 17 reported negative mutagenicity. The remaining two studies only reported very weak mutagenic effects. The authors related this phenomenon to the degree of nanoparticle uptake by the bacterial cells, which is likely to be less than in mammalian cells, due to their incapability to cross the cell wall.

Based on our previous work on the electrostatic interactions between nanoparticles and *Escherichia coli* (Pagnout et al., 2012), we believe that the lack of interactions between nanoparticles and bacteria could make the bacterial reverse mutation test unsuitable for nanoparticle mutagenicity assessment due to the use of an inadequate exposure medium. We also think that a pre-exposure step in a simple saline solution at a pH value below the nanoparticle isoelectric points, which would allow interactions between bacterial cells and nanoparticles, can strongly improve the accuracy of the test. NP-TiO_2 aggregation and electrophoretic mobilities of both bacterial cells and nanoparticles in the exposure medium of the bacterial reverse mutation assay (AM) and in NaCl saline solution (SS) were conducted.

3.3. NP-TiO_2 aggregation in exposure media

Nanoparticle aggregation measurements were performed in both AM and SS (Fig. 1). No statistical difference was found between TiO_2-P25 aggregate sizes in the two exposure media. Their average sizes were found to be approximately 800 nm in AM and 700 nm in SS. Regarding TiO_2-NA, the average size of aggregates was found to be approximately 3800 nm in AM and only 67 nm in the SS. Significant differences between the average aggregate size in the two media were also observed for TiO_2-TLB, which had sizes of more than 1300 nm in the AM and 730 nm in SS.

Nanoparticle aggregation in exposure media can be explained in the framework of the DLVO theory (named after Derjaguin, Landau, Verwey and Overbeek) of colloidal stability, which considers the balance between the repulsive electrostatic interactions and

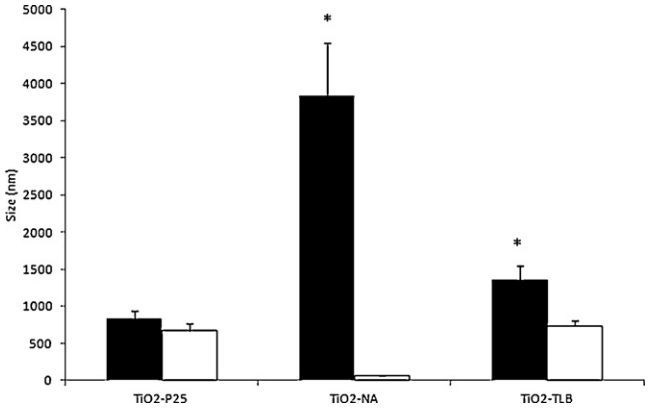

Fig. 1. Average sizes of the NP-TiO_2 aggregates in the Ames medium (black bars) and in the saline solution (white bars) after 5 min. Aggregation experiments were performed independently four times for each NP-TiO_2 in both media. The statistical differences are determined by the Wilcoxon–Mann–Whitney test with a p-value <0.05 (*).

the attractive Van der Waals forces (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). If electrostatic repulsion dominates, the particles repel each other and the nanoparticle suspension is well dispersed. Reversely, if Van der Waals forces dominate, nanoparticles experience mutual attraction and aggregate. The pH, electrolyte concentration, and ion valence determine the strength of electrostatic forces. AM contains polyvalent ions and several organic compounds (glucose, biotin, histidine and bromocresol purple) that can screen charges of the nanoparticles. Moreover, the pH value of AM (pH 7.4) is close to the isoelectric point of NP-TiO_2 (6.8 for TiO_2-P25, 6.5 for TiO_2-NA and between 7 and 8 for TiO_2-TLB). In these conditions, nanoparticles quickly aggregate as soon as they are introduced to AM and then cannot properly interact with bacterial cells during the bacterial reverse mutation assay, potentially leading to the underestimation of their mutagenic potential. Conversely, SS contains exclusively monovalent ions and has a pH value (pH 5.5) further away from the isoelectric points of NP-TiO_2, thereby leading to reduced nanoparticle aggregation compared to AM.

3.4. Bacterial and NP-TiO_2 electrophoretic mobility in exposure media

Electrophoretic mobility of both NP-TiO_2 and *S. typhimurium* strains in AM and in SS are presented in Table 2. Regardless of the medium considered, bacterial strains are always negatively charged. Depending on the strain, electrophoretic mobilities vary from −0.27 μm/s/V/cm (strain TA100) to −2.30 μm/s/V/cm (strain TA98) in AM and from −0.79 μm/s/V/cm (strain TA100) to −4.87 μm/s/V/cm (strain TA97a) in SS. These results are consistent with those previously obtained by several authors showing that at

Table 2
Electrophoretic mobilities of the *Salmonella typhimurium* strains and NP-TiO_2.

		Mobility in the fluctuation test medium (μm/s/V/cm)	Mobility in the saline solution (μm/s/V/cm)
S. typhimurium strains	TA97a	−2.07	−4.87
	TA98	−2.30	−4.74
	TA100	−0.27	−0.79
	TA102	−0.92	−1.63
NP-TiO_2	TiO_2-P25	−2.94	+2.42
	TiO_2-NA	−2.60	+3.19
	TiO_2-TLB	−2.70	+1.99

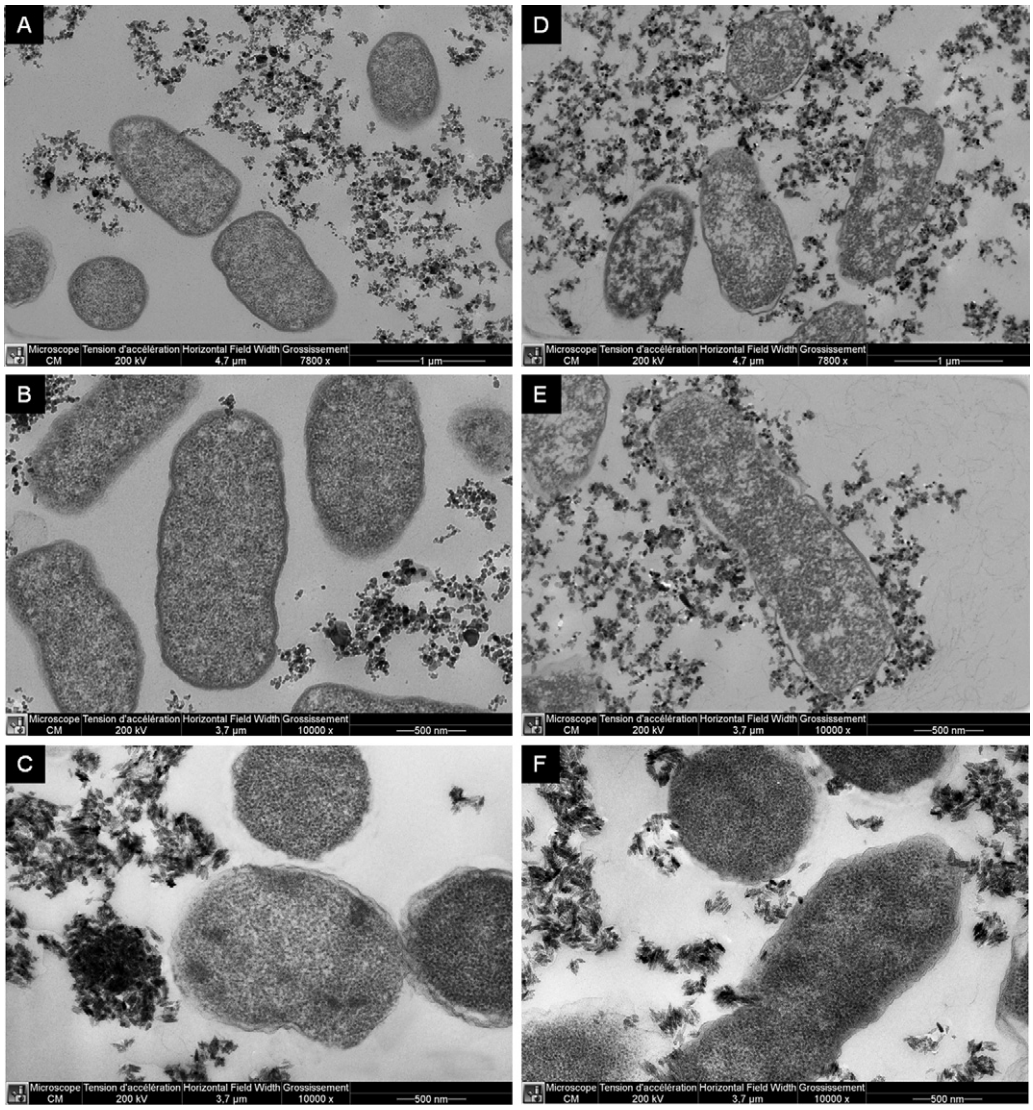

Fig. 2. Transmission electron microscopy images of *Salmonella typhimurium* TA 102 after TiO_2-P25 (A, B, D, and E) and TiO_2-TLB exposure (C and F) in the exposure medium of the bacterial reverse mutation test (A–C) and in the NaCl saline solution (D–F).

environmental pH, most bacterial cell wall surfaces are negatively charged due to the presence of several functional groups (carboxyl, phosphate and hydroxyl) (Blake et al., 1999; Kiwi and Nadtochenko, 2004; Rincon and Pulgarin, 2004).

Conversely, in AM, the NP-TiO_2, which we previously showed aggregated, have strongly negative electrophoretic mobilities (−2.94 μm/s/V/cm for TiO_2-P25, −2.60 μm/s/V/cm for TiO_2-NA and −2.70 μm/s/V/cm for TiO_2-TLB) (Table 2). In contrast, all NP-TiO_2 exhibited net positive charges in SS (+2.42 μm/s/V/cm for TiO_2-P25, +3.19 μm/s/V/cm for TiO_2-NA and +1.99 μm/s/V/cm for TiO_2-TLB).

Consequently, electrostatic forces between negatively charged bacterial cells and nanoparticles in AM would be repulsive; whereas in SS, these forces would be attractive due to the nanoparticle charge inversion, leading to higher interactions. This is supported by the TEM observations showing that nanoparticles strongly adsorb on the surface of the bacterial cell wall in SS, whereas this is not the case in AM (Fig. 2).

3.5. Mutagenicity assay using the modified fluctuation test

The conventional fluctuation test was modified as described in Section 2. A pre-exposure step of 0.1 h, 10 h or 20 h in SS was performed before testing to improve interactions between NP-TiO_2 and bacterial cells. Results presented in Table 3 revealed high mutagenicity of NP-TiO_2 tested with *S. typhimurium* strain TA102. For TiO_2-P25, positive responses were obtained at higher nanoparticle concentrations after 0.1, 10 h or 20 h of pre-exposure in SS. The strongest response was observed with 100 mg/L of TiO_2-P25 after 20 h pre-exposure with 96 positive wells. TiO_2-P25 mutagenicity was also detected to a lesser extent with strain TA98 at 100 mg/L of nanoparticles after 0.1 and 10 h pre-exposure in SS and at 10 and 100 mg/L after 20 h pre-exposure in SS. For TiO_2-NA, mutagenicity was exclusively detected with strain TA102. A stronger response was obtained with 1 and 10 mg/L of nanoparticles after 10 h or 20 h pre-exposure of 96 positive wells. Table 3 also indicated that NP-TiO_2, which had an average size much smaller than TiO_2-P25 in SS (∼67 nm for the TiO_2-NA and ∼700 nm for the TiO_2-P25), induced toxicity in strains TA97a, TA98 and TA102 at higher concentrations (numbers of revertants obtained after exposure lower than the number of spontaneous revertants obtained with the control). For the TiO_2-byproduct (TiO_2-TLB), as for all NP-TiO_2, mutagenicity was detected with strain TA102 at 100 mg/L after 0.1 h pre-exposure, 10 and 100 mg/L after 10 h pre-exposure, and 1, 10 and 100 mg/L after 20 h pre-exposure in SS. To insure that the mutagenicity observed

Table 3
Number of revertants obtained with the modified fluctuation test using the SS as pre-exposure medium.

	Pre-exposure time (h)	Pre-exposure concentration (mg/L)[a]	*Salmonella typhimurium* strains[b]			
			TA97a	TA98	TA100	TA102
TiO_2-P25	0.1	0	43/32/43	12/5	10/14/11	68/41/70
		1	34/35/44	17/2	10/12/8	67/61/69
		10	30/35/33	12/10	5/21/18	71/68/65
		100	35/42/32	43/81	19/10/17	95/71/68
	10	0	23/36	17/7	13/15/18	65/45/63
		1	30/26	13/8	13/5/11	64/57/67
		10	36/35	14/14	11/7/12	66/64/76
		100	36/38	76/59	11/8/15	96/65/94
	20	0	22/27	11/14/12	14/18	68/40/74
		1	28/24	32/22/23	12/13	64/74/82
		10	37/28	63/22/45	19/10	67/57/87
		100	38/22	89/94/88	13/11	96/96/96
TiO_2-NA	0.1	0	37/33	5/21	13/10	68/66/70
		1	41/36	6/27	12/17	84/65/96
		10	31/35	7/18	15/15	92/79/96
		100	26/31	4/10	14/15	96/87/90
	10	0	29/30	21/18	16/31	69/76
		1	32/32	12/11	15/24	96/96
		10	22/23	13/9	10/28	96/96
		100	17/18	1/0	10/17	37/16
	20	0	32/34	7/13	15/17	59/72/64
		1	40/34	9/11	11/17	92/96/96
		10	37/32	4/6	12/18	96/96/96
		100	15/3	4/0	24/14	30/72/66
TiO_2-TLB	0.1	0	25/25/22	6/26	21/10	67/61/66
		1	20/25/31	8/17	12/14	71/68/72
		10	17/35/33	4/24	19/18	81/66/70
		100	14/35/32	6/14	19/14	95/77/58
	10	0	31/24	19/15	33/25	65/68
		1	25/27	17/18	43/22	72/70
		10	37/31	13/17	50/30	78/80
		100	21/31	14/19	43/31	77/82
	20	0	18/25	4/25	16/10	68/63/76
		1	16/36	3/11	14/8	96/70/90
		10	18/35	4/16	19/10	90/70/96
		100	22/31	7/14	40/20	96/84/96
Positive controls						
9-AA		10	96/96/96			
2-NF		0.4		95/96/93		
SA		0.025			85/96/96	
CH		3.5				96/96/96

[a] NP-TiO_2 concentrations in SS, giving final concentrations of 0, 0.025, 0.25 and 2.5 mg/L in the fluctuation test.
[b] Numbers of revertants statically different (Chi-square analysis (Gilbert, 1980)) from the number of spontaneous revertants obtained with the control are underlined. Experiments were performed independently twice and one 96-well microplate was used for each concentration. When these experiments gave contradictory results, the experiment was repeated a third time. Mutagenicity of NP-TiO_2 is considered when the tests showed positive responses at least twice (results highlighted in gray).

with the TiO_2-TLB was not related to other elements present in the byproduct, such as aluminum or PDMS residues released during the nanocomposite alteration process (Labille et al., 2010), the fluctuation test was also performed on the supernatant fraction obtained after centrifugation (10,000 × *g* for 5 min at 4 °C) and filtration using a 25 nm membrane filter. Regardless of the strain used, results showed no mutagenicity of this fraction (data not shown).

An additional set of experiments was also done using the AM instead of the SS as a pre-exposure medium to support the hypothesis of an exposure medium-effect in the NP-TiO_2 genotoxicity observed with the modified fluctuation test, but not with the conventional one. Indeed, the pre-exposure step of the modified fluctuation test is performed in 100 mL flasks at 20 °C under agitation, whereas the conventional test is performed in 96-well microtiter plates at 37 °C without agitation. Results obtained after 20 h of bacterial cell pre-exposure to TiO_2-P25, TiO_2-NA and TiO_2-TLB in the AM showed no positive response (Supplementary data Table S1), supporting the initial hypothesis that a pre-exposure step in a simple saline solution (NaCl 10 mM, pH 5.5) improved the interactions between nanoparticles and bacterial cells and the accuracy of the fluctuation test.

To the best of our knowledge, this study is the first to report NP-TiO_2 mutagenic potential by the use of the bacterial reverse mutation test. The mechanisms by which these nanoparticles can cause mutagenicity have not been clearly elucidated, but several pathways have been examined. Some studies have shown that nanoparticles can potentially cross the bacterial cell wall and locate to the cytoplasm (Sondi and Salpotek-Sondi, 2004; Xu et al., 2004; Brayner et al., 2006; Huang et al., 2000; Kumar et al., 2011). In this case, mutagenicity caused by direct or indirect interaction between nanoparticles and DNA, and/or its regulatory apparatus (primary mechanisms), could be a first hypothesis. However, in our study, mutagenicity was observed even with large-sized or aggregated nanoparticles. Moreover, TEM observations of the *S. typhimurium* strains after exposure did not show the presence of NP-TiO_2 inside the cells (>50 cells observed per bacterial strain)

(Fig. 2). Another hypothesis of indirect genotoxic mechanisms, resulting from reactive oxygen radical induction that may damage lipids, proteins or DNA, is more likely (secondary mechanisms). This idea is supported by the high sensitivity of the TA102 strain in detecting NP-TiO_2 mutagenicity. Indeed, this strain was specifically developed by Levin et al. (1982) to detect a variety of oxidative mutagens. However, even though there is ample evidence for reactive oxygen species (ROS) formation when TiO_2 is exposed to UV light (Matsunaga et al., 1985; Konaka et al., 1999; Hirakawa et al., 2004; Gurr et al., 2005), the ROS production without UV irradiation is less documented and somewhat more controversial. Fenoglio et al. (2009) showed direct evidence that NP-TiO_2 are active in the generation of free radicals, including oxygenated free radicals and carbon-centered radicals, causing cleavage of C—H bonds in a model organic molecules. These reactions can occur even in the dark and could serve as the first step of oxidative damage of biological molecules such as polyunsaturated fatty acids to form harmful aldehydes that can trigger longer periods of damage to proteins and other molecules (i.e. lipid peroxidation). Such redox interactions could in turn generate more free radicals that perpetuate the formation of highly reactive epoxides that could further compromise the integrity of the cell membrane and damage DNA (Lyon et al., 2007; Cabiscol et al., 2000). Gou et al. (2010) recently highlighted this mechanism by analysis of the *E. coli* whole-cell-array stress genes exposed to NP-TiO_2 in the dark. The mechanisms by which lipid peroxidation may be involved in mutagenicity are twofold. The lipid-derived radicals and their breakdown products, such the malondialdehyde (MDA) which is a major dead-end product, can interact with DNA, forming intra- and inter-strand DNA crosslinks and cyclic base adducts. MDA is known to revert frameshift and base-pair substitution mutations in *S. typhimurium* (Marnett et al., 1985; Basu and Marnett, 1983), which could explain the TiO_2-P25 mutagenicity detected with both TA102 and TA98 strains. Lipid peroxidation products are also known to affect the cell indirectly by altering cell metabolism, binding to cellular proteins, inactivating macromolecule synthesis and depleting cellular antioxidants such as glutathione (Howden and Faux, 1996). In regards to mammalian/human cell studies, it was shown that, even in the dark, NP-TiO_2 induces oxidative stress-mediated toxicity in many cell types (Gurr et al., 2005; Wang et al., 2007; Hussain et al., 2010; Shukla et al., 2011; Meena et al., 2012; Saquib et al., 2012) and the lipid peroxidation was also suspected as the underlying mechanism of this genotoxicity. On human bronchial epithelial cell line (BEAS-2B), Gurr et al. (2005) reported that in the absence of photoactivation, the anatase NP-TiO_2 (10 and 20 nm) can induce an increase of the cellular MDA level, oxidative DNA damage, and increase cellular nitric oxide and hydrogen peroxide levels. Shukla et al. (2011) that NP-TiO_2 can induce micronucleus formation, significant reduction in glutathione with a concomitant increase in lipid hydroperoxide and ROS generation in exposed human epidermal cells.

4. Conclusions

The booming demand for nanoparticles has spurred significant public alarm about their possible adverse effects. TiO_2 nanoparticles, like several other nanoparticles, have been listed by the OECD as a priority nanomaterial for immediate testing. Several studies have been published, in which the bacterial reverse mutation test (OECD test guideline 471) was utilized for genotoxicological analysis of nanoparticles. Most have reported negative mutagenicity of nanoparticles, whereas the same nanoparticles have largely been found to have positive genotoxic responses in other in vitro mammalian cell test systems (Doak et al., 2012).

In the present study, we showed that the medium used for bacterial exposure to nanoparticles in the bacterial reverse mutation test is not suitable. Indeed, this medium prevents electrostatic interactions between bacterial cells and nanoparticles and promotes the aggregation of the latter. It was also shown that a simple pre-exposure step in a low ionic-strength solution, at a pH below the nanoparticle isoelectric points (NaCl 10 mM, pH 5.5), could strongly improve the accuracy of the test. Thus, based on these improvements, we highlighted the mutagenicity of the two engineered NP-TiO_2 tested and a NP-TiO_2 byproduct derived from a nanocomposite that is commonly used in sunscreens and purportedly inert.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the French National Agency for Research (ANR CESA AgingNano&Troph) and by the Conseil Régional de Lorraine. The authors thank the Laboratoire Environnement et Minéralurgie (LEM – CNRS UMR 7569) for giving us an access to their zetaphoremeter. They also thank Jaafar Ghanbaja from the SCMEM (Nancy – France) for the TEM observations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2012.09.012.

References

Aeschbacher, H.U., Wolleb, U., Porchet, L., 1987. Liquid preincubation mutagenicity test for foods. Journal of Food Safety 8, 167–177.
Ames, B.N., Gurney, E.G., Miller, J.A., Bartsch, H., 1972. Carcinogens as frameshift mutagens: metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proceeding of the National Academy of Sciences of the United States of America 69, 3128–3132.
Ames, B.N., Lee, F.D., Durston, W.E., 1973a. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proceeding of the National Academy of Sciences of the United States of America 70, 782–786.
Ames, B.N., Durston, W.E., Yamasaki, E., Lee, F.D., 1973b. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proceeding of the National Academy of Sciences of the United States of America 70, 2281–2285.
Auffan, M., Pedeutour, M., Rose, J., Masion, A., Ziarelli, F., Borschneck, D., Chaneac, C., Botta, C., Chouand, P., Labille, J., Bottero, J.Y., 2010. Surface structural degradation of a TiO_2-based nanomaterial used in cosmetics. Environmental Science and Technology 44, 2689–2694.
Balasubramanyam, A., Sailaja, N., Mahboob, M., Rahman, M.F., Misra, S., Hussain, S.M., Grover, P., 2009. Evaluation of genotoxic effects of oral exposure to aluminum oxide nanomaterials in rat bone marrow. Mutation Research 676, 41–47.
Basu, A.K., Marnett, L.J., 1983. Unequivocal demonstration that malondialdehyde is a mutagen. Carcinogenesis 4, 331–333.
Bigorgne, E., Foucaud, L., Lapied, E., Labille, J., Botta, C., Sirguey, C., Falla, J., Rose, J., Joner, E.J., Rodius, F., Nahmani, J., 2011. Ecotoxicological assessment of TiO_2 byproducts on the earthworm *Eisenia fetida*. Environmental Pollution 159, 2698–2705.
Blake, D., Maness, P., Huang, Z., Wolfrum, E., Huang, J., Jacoby, W.A., 1999. Application of the photocatalytic chemistry of titanium dioxide to disinfection and the killing of cancer cells. Separation and Purification Methods 28, 1–50.
Brayner, R., Ferrari-Iliou, R., Brivois, N., Djediat, S., Benedetti, M.F., Fivet, F., 2006. Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Letters 6, 866–870.
Cabiscol, E., Tamarit, J., Ros, J., 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. International Microbiology 3, 3–8.
Colvin, V.L., 2003. The potential environmental impact of engineered nanomaterials. Nature Biotechnology 21, 1166–1170.
Davoren, M.D., 2005. Luminescent bacterial biosensors for the rapid detection of toxicants. In: Water Encyclopedia, pp. 453–458.
Derjaguin, B.V., Landau, L.D., 1941. Acta Physicochimica URSS 14, 733–742.
Di Virgilio, A.L., Reigosa, M., Arnal, P.M., Fernandez Lorenzo de Mele, M., 2010. Comparative study of the cytotoxic and genotoxic effects of titanium oxide and aluminium oxide nanoparticles in Chinese hamster ovary (CHO-K1) cells. Journal of Hazardous Materials 177, 711–718.

Doak, S.H., Manshian, B., Jenkins, G.J.S., Singh, N., 2012. In vitro genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. Mutation Research 745, 104–111.
Environment Canada, 1993. Protocole-Test de fluctuation. Laboratoire C&P (CSL), Montreal, Canada.
Felton, J.S., Wu, R.W., 2003. Ames/Salmonella assay: a bacterial test for mutagens. In: Encyclopedia of Life Sciences. Nature Publishing Group, John Wiley & Sons Ltd, Chichester.
Fenoglio, I., Greco, G., Livraghi, S., Fubini, B., 2009. Non-UV-induced radical reactions at the surface of TiO_2 nanoparticles that may trigger toxic responses. Chemistry 15, 4614–4621.
Gilbert, R.I., 1980. The analysis of fluctuation tests. Mutation Research 74, 283–289.
Gleiche, M., Hoffschulz, H., Lenhert, S., 2006. Nanotechnology in Consumer Products, Nanoforum Report: Nanotechnology in Consumer Products, pp. 1–31.
Gou, Z., Onnis-Hayden, A., Gu, A.Z., 2010. Mechanistic toxicity assessment of nanomaterials by whole-cell-array stress genes expression analysis. Environmental Science and Technology 44, 5964–5970.
Green, M.H.L., Muriel, W.J., Bridges, B.A., 1976. Use of a simplified fluctuation test to detect low levels of mutagens. Mutation Research 38, 33–42.
Gurr, J., Wang, A.S.S., Chen, C., Jan, K., 2005. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. Toxicology 213, 66–73.
Hirakawa, K., Mori, M., Yoshida, M., Oikawa, S., Kawanishi, S., 2004. Photo-irradiated titanium dioxide catalyzes site specific DNA damage via generation of hydrogen peroxide. Free Radical Research 38, 439–447.
Howden, P.J., Faux, S.P., 1996. Glutathione modulates the formation of 8-hydroxydeoxyguanosine in isolated DNA and mutagenicity in *Salmonella typhimurium* TA100 induced by mineral fibres. Carcinogenesis 17, 2275–2277.
Huang, Z., Maness, P.-C., Blake, D.M., Wolfrum, E.J., Smolinski, S.L., Jacoby, W.A., 2000. Bactericidal mode of titanium dioxide photocatalysis. Journal of Photochemistry and Photobiology A: Chemistry 130, 163–170.
Hubbard, S.A., Green, M.H.L., Gatehouse, D., Bridges, J.W., 1984. The fluctuation test in bacteria. In: Kilbey, B.J., Legator, M., Nichols, W., Ramel, C. (Eds.), Handbook of Mutagenicity Test Procedures. , 2nd edition. Elsevier, Amsterdam, New York, Oxford, pp. 141–161.
Hussain, S., Thomassen, L.C., Ferecatu, I., Borot, M.C., Andreau, K., Martens, J.A., Fleury, J., Baeza-Squiban, A., Marano, F., Boland, S., 2010. Carbon black and titanium dioxide nanoparticles elicit distinct apoptotic pathways in bronchial epithelial cells. Particle and Fibre Toxicology 7, 10.
Kirkland, D., Reeve, L., Gatehouse, D., Vanparys, P., 2011. A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. Mutation Research 721, 27–73.
Kiwi, J., Nadtochenko, V., 2004. New evidence for TiO_2 photocatalysis during bilayer lipid peroxidation. The Journal of Physical Chemistry B 108, 17675–17684.
Konaka, R., Kasahara, E., Dunlap, W.C., Yamamoto, Y., Chen, K.C., Inoue, M., 1999. Irradiation of titanium dioxide generates both singlet oxygen and superoxide anion. Free Radical Biology and Medicine 27, 294–300.
Kumar, A., Pandey, A.K., Singh, S.S., Shanker, R., Dhawan, A., 2011. Cellular uptake and mutagenic potential of metal oxide nanoparticles in bacterial cells. Chemosphere 83, 1124–1132.
Labille, J., Feng, J., Botta, C., Borschneck, D., Sammut, M., Cabie, M., Auffan, M., Rose, J., Bottero, J.Y., 2010. Aging of TiO_2 nanocomposites used in sunscreen, dispersion and fate of the degradation products in aqueous environment. Environmental Pollution 158, 3482–3489.
Levin, D.E., Hollstein, M., Christman, M.F., Schwiers, E.A., Ames, B.N., 1982. A new Salmonella tester strain (TA102) with AT base pairs at the site of mutation detects oxidative mutagens. Proceeding of the National Academy of Sciences of the United States of America 79, 7445–7449.
Lyon, D.Y., Thill, A., Rose, J., Alvarez, P.J.J., 2007. Ecotoxicological impacts of nanomaterials. In: Wiesner, M., Bottero, J.Y. (Eds.), Environmental Nanotechnology: applications and impacts of nanomaterials. McGraw Hill, New York, pp. 445–479.
Marnett, L.J., Hurd, H.K., Hollstein, M.C., Levin, D.E., Esterbauer, H., Ames, B.N., 1985. Naturally occurring carbonyl compounds are mutagens in Salmonella tester strain TA104. Mutation Research 148, 25–34.
Maron, D.M., Ames, B.N., 1983. Revised methods for the Salmonella mutagenicity test. Mutation Research 113, 173–215.
Matsunaga, T., Tomoda, R., Nakajima, T., Wake, H., 1985. Photoelectrochemical sterilization of microbial cells by semiconductor powders. FEMS Microbiology Letters 29, 211–214.
McCann, J., Choi, E., Yamasaki, E., Ames, B.N., 1975. Detection of carcinogens in the Salmonella/microsome test. Assay of 300 chemicals. Proceeding of the National Academy of Sciences of the United States of America 72, 5135–5139.
McPherson, F.M., Nestmann, E.R., 1990. The simultest approach for testing mutagens in the salmonella microtitre fluctuation assay. Environmental and Molecular Mutagenesis 16, 21–25.
Meena, R., Rani, M., Pal, R., Rajamani, P., 2012. Nano-TiO_2-induced apoptosis by oxidative stress-mediated DNA damage and activation of p53 in human embryonic kidney cells. Applied Biochemistry and Biotechnology 167, 791–808.
Mortelmans, K., Zeiger, E., 2000. The Ames Salmonella/microsome mutagenicity assay. Mutation Research 455, 29–60.
Nel, A., Xia, T., Madler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311, 622–627.
Ng, C.T., Li, J.J., Bay, B.H., Lanry Yung, L.Y., 2010. Current studies into the genotoxic effects of nanomaterials. Journal of Nucleic Acids 2010, 1–12.
OECD, 2008. List of Manufactured Nanomaterials and List of Endpoints for Phase One of the OECD Testing Programme. Series on the Safety of Manufactured Nanomaterials 27. Organization for Economic Cooperation and Development, Paris, France.
Osman, I.F., Baumgartner, A., Cemeli, E., Fletcher, J.N., Anderson, D., 2010. Genotoxicity and cytotoxicity of zinc oxide and titanium dioxide in HEp-2 cells. Nanomedicine 5, 1193–1203.
Pagnout, C., Jomini, S., Dadhwal, M., Caillet, C., Thomas, F., Bauda, P., 2012. Role of electrostatic interactions in the toxicity of titanium dioxide nanoparticles toward *Escherichia coli*. Colloids and Surfaces B: Biointerfaces 92, 315–321.
Pan, X., Redding, J.E., Wiley, P.A., Wen, L., McConnell, J.S., Zhang, B., 2010. Mutagenicity evaluation of metal oxide nanoparticles by the bacterial reverse mutation assay. Chemosphere 79, 113–116.
Qureshi, A.A., Coleman, R.N., Paran, J.H., Liu, D., 1984. In: Butka, B.J. (Ed.), Toxicity Screening Procedures using Bacterial Systems. Marcel Dekker Inc., New York and Basel, pp. 1–22.
Rincon, A.G., Pulgarin, C., 2004. Field solar *E. coli* inactivation in the absence and presence of TiO_2: is UV solar dose an appropriate parameter for standardization of water solar disinfection? Solar Energy 77, 635–648.
Saquib, Q., Al-Khedhairy, A.A., Siddiqui, M.A., Abou-Tarboush, F.M., Azam, A., Musarrat, J., 2012. Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. Toxicology In Vitro 26, 351–361.
Shi, Y., Zhang, J.H., Jiang, M., Zhu, L.H., Tan, H.Q., Lu, B., 2010. Synergistic genotoxicity caused by low concentration of titanium dioxide nanoparticles and p,p′-DDT in human hepatocytes. Environmental and Molecular Mutagenesis 51, 192–204.
Shukla, R.K., Kumar, A., Pandey, A.K., Singh, S.S., Dhawan, A., 2011. Titanium dioxide nanoparticles induce oxidative stress-mediated apoptosis in human keratinocyte cells. Journal of Biomedical Nanotechnology 7, 100–101.
Singh, N., Manshian, B., Jenkins, G.J.S., Griffiths, S.M., Williams, P.M., Maffeis, T.G.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterial. Biomaterials 30, 3891–3914.
Sondi, I., Salpotek-Sondi, B., 2004. Silver nanoparticles as antimicrobial agent, a case study on *E. coli* as a model for Gram-negative bacteria. Journal of Colloid and Interface Science 275, 177–182.
Strum, J.M., Wicken, J., Stanbury, J.R., Karnowsky, M.J., 1971. Appearance and function of endogenous peroxidase in fetal rat tyroid. Journal of Cell Biology 51, 162–175.
Tennant, R.W., Margolin, B.H., Shelby, M.D., Zeiger, E., Haseman, J.K., Spalding, J., Resnick, W., Stasiewicz, M., Anderson, B., Minor, R., 1987. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. Science 236, 933–941.
The Royal Society and the Royal Academy of Engineering, 2003. Environmental Applications and Impacts of Nanotechnology: Summary of Evidence Presented to Nanotechnology Working Group.
The Royal Society, 2004. Nanoscience and Nanotechnologies: Opportunities and Uncertainties., http://www.nanotec.org.uk/finalReport.htm
The White House, 2000. National Nanotechnology Initiative: Leading to the Next Industrial Revolution. The White House Office of the Press Secretary, Washington, DC.
The Woodrow Wilson Database, 2011. http://www.nanotechproject.org
Thompson, E.D., Melampy, P.J., 1981. An examination of the quantitative suspension assay for mutagenesis with strains of *Salmonella typhimurium*. Environmental Mutagenesis 3, 453–465.
Verwey, E.J.W., Overbeek, J.T.G., 1948. Theory of the Stability of Lyophobic Colloids. Elsevier, Amsterdam.
Wang, J.J., Sanderson, B.J., Wang, H., 2007. Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. Mutation Research 628, 99–106.
Warheit, D.B., Hoke, R.A., Finlay, C., Donner, E.M., Reed, K.L., Sayes, C.M., 2007. Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. Toxicology Letters 171, 99–110.
Xu, X.H., Brownlow, W.J., Kyriacou, S.V., Wan, Q., Viola, J.J., 2004. Real-time probing of membrane transport in living microbial cells using single nanoparticles optics and living cell imaging. Biochemistry 43, 10400–10413.
Zeiger, E., 1998. Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: premises, promises, and performance. Regulatory Toxicology and Pharmacology 28, 85–95.