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Genotoxicity evaluation of titanium dioxide nanoparticles using the Ames test and Comet assay§

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ABSTRACT: Titanium dioxide nanoparticles (TiO_2-NPs) are being used increasingly for various industrial and consumer products, including cosmetics and sunscreens because of their photoactive properties. Therefore, the toxicity of TiO_2-NPs needs to be thoroughly understood. In the present study, the genotoxicity of 10nm uncoated sphere TiO_2-NPs with an anatase crystalline structure, which has been well characterized in a previous study, was assessed using the *Salmonella* reverse mutation assay (Ames test) and the single-cell gel electrophoresis (Comet) assay. For the Ames test, *Salmonella* strains TA102, TA100, TA1537, TA98 and TA1535 were preincubated with eight different concentrations of the TiO_2-NPs for 4 h at 37 °C, ranging from 0 to 4915.2 μg per plate. No mutation induction was found. Analyses with transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (EDS) showed that the TiO_2-NPs were not able to enter the bacterial cell. For the Comet assay, TK6 cells were treated with 0–200 μg ml^{-1} TiO_2-NPs for 24 h at 37 °C to detect DNA damage. Although the TK6 cells did take up TiO_2-NPs, no significant induction of DNA breakage or oxidative DNA damage was observed in the treated cells using the standard alkaline Comet assay and the endonuclease III (*Endo*III) and human 8-hydroxyguanine DNA-glycosylase (hOGG1)-modified Comet assay, respectively. These results suggest that TiO_2-NPs are not genotoxic under the conditions of the Ames test and Comet assay. Published 2012. This article is a US Government work and is in the public domain in the USA.

Keywords: titanium dioxide nanoparticles; Ames test; Comet assay; mutations; genotoxicity

Introduction

The industrial use of titanium dioxide (TiO_2) has been commonplace for years. Until recently, the use had been limited to coarse and fine (both diameters larger than 100 nm) TiO_2 particles. Both coarse and fine particles of TiO_2 have been investigated and declared biologically inert in humans and animals (Chen and Fayerweather 1988; Hart and Hesterberg 1998; Bernard *et al*., 1990). However, TiO_2 nanoparticles (TiO_2-NPs) are increasingly being used in pharmaceuticals and cosmetics because of their colorless and photoactive properties (Cai *et al*., 1992; Kubota *et al*., 1994). TiO_2-NPs are distinctive from the larger TiO_2 particles owing to their unique properties derived from their small sizes (less than 100 nm), which call into question their biological inertness. Research into the toxicity of TiO_2-NPs has uncovered evidence for pulmonary inflammation, fibrosis and DNA damage (Afaq *et al*., 1998; Baggs *et al*., 1997; Rahman *et al*., 2002). Moreover, it has been suggested that TiO_2-NPs are small enough to penetrate beyond the human stratum corneum and then affect the immune system (Gurr *et al*., 2005). There is also potential for TiO_2-NPs to induce reactive oxygen species (ROS) thus causing oxidative DNA damage especially when the TiO_2-NPs are irradiated with UV/visible light (Kang *et al*., 2008; Nakagawa *et al*., 1997). TiO_2-NPs are possibly carcinogenic to humans (Group 2B) based on sufficient evidence in experimental animals and inadequate evidence from epidemiology studies according to the report from the International Agency for Research on Cancer (IARC) (IARC, 2009). The National Institute for Occupational Safety and Health (NIOSH) recently (NIOSH 2011) also concluded that ultrafine (nano-sized) TiO_2 is a potential occupational carcinogen, which acts through a secondary genotoxicity mechanism primarily related to particle size and surface area.

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While many different types of nanoparticles have been tested and found to have genotoxic effects, there remain clear inconsistencies in the reported results. Several recent review articles examining the current literature suggest a number of factors that could contribute to the inconsistent results (Doak *et al*., 2009; Gonzales *et al*., 2008; Landsiedel *et al*., 2009; Singh *et al*., 2009). First, physicochemical characterization of the nanoparticles being evaluated was found to be limited in many early studies. These inadequacies make it impossible to accurately determine important characteristics of the nanostructures under investigation. Thus, conflicting results reported from different studies could have arisen from variability in characteristics of the test materials, such as size, shape and crystalline structure. Second, cell uptake of nanoparticles was not investigated in many studies; hence, negative results could have been caused by a lack of cellular uptake rather than an absence of genotoxicity. Third, evaluation of the potential genotoxicity of nanoscale materials should include a set of genotoxicity assays with different genotoxicity endpoints, which could provide a broad characterization of the potential genotoxicity of nanomaterials. Finally, the manner in which genetic damage is measured varies among different test systems; and some assays could be ill-suited for evaluating particular types of nanomaterials. For example, bacterial mutagenicity-based assays may not be suitable for detecting the genotoxicity of nanoparticles because the prokaryotic cells may lack critical abilities that eukaryotic cells maintain. One such difference may be found in the decreased penetrability of the prokaryotic cell wall for nanoparticles compared with the eukaryotic cell membrane, which can perform endocytosis (Warheit *et al*., 2007).

As with many other nanomaterials, controversial results have been reported on the genotoxicity of TiO_2-NPs (Table 1). Some studies showed that TiO_2-NPs were genotoxic, such as studies measuring micronucleus formation and DNA breakage (Kang *et al*., 2008), chromosomal segregation (Huang *et al*., 2009), DNA damage (Gopalan *et al*., 2009; Wang *et al*., 2007) and gene mutation (Wang *et al*., 2007). Others concluded that TiO_2-NPs were not genotoxic in the Comet assay (Bhattacharya *et al*., 2009; Gerloff *et al*., 2009; Nakagawa *et al*., 1997), *in vivo* 8-oxoguanine detection (Rehn *et al*., 2003) and Ames test and an *in vitro* mammalian chromosome aberration test with Chinese hamster ovary cells (Warheit *et al*., 2007). It is possible that the inconsistency in the literature is due to different types of TiO_2-NPs tested, the study design and/or inadequate characterization of the physical–chemical properties of the nanomaterials.

In light of these problems, it was the main goal of the present study to evaluate whether or not well-characterized 10 nm TiO_2-NPs can induce mutations in the Ames test, an assay measuring the ability of the test material to induce base pair substitutions and frame-shift mutation (Ames *et al*., 1973; Mortelmans and Zeiger 2000), and DNA single- and double-strand breaks (SSB/DSB), alkali-labile sites (ALS), DNA-DNA/DNA-protein cross-linking in the standard and enzyme-modified Comet assays in human lymphoblastoid TK6 cells, a test detecting DNA strand breakage resulting from a test material (Henderson *et al*., 1998; Singh *et al*., 1988; Tice *et al*., 2000). In addition, the cellular uptake of TiO_2-NPs into the bacterial and TK6 cells was examined with a transmission electron microscope (TEM) to assess the assays' suitability for evaluating the nanoparticles.

Materials and Methods

Characterization of TiO_2-NPs

The TiO_2-NPs were synthesized at the Nanotechnology Center, University of Arkansas, Little Rock, AR, USA, based on a previously described method (Sugimoto *et al*., 2002).

The size distribution, aggregation in buffer and treatment medium, and characteristics were described in our previous study (Sadiq *et al*., 2012). The dry size distribution of the TiO_2-NPs was approximately 10×30 nm and the nanoparticles had an anatase crystalline structure. They were heavily aggregated not only in dry powder (Fig. 1) but also in solutions (the aggregated sizes was approximately 130–170 nm) (Sadiq *et al*., 2012).

Cellular Uptake of the TiO_2-NPs in Bacteria and TK6 Cells

Salmonella typhimurium TA100 cells were treated with 200 μg TiO_2-NPs ml^{-1} in nutrient broth II for 4 h and TK6 cells were treated with 200 μg TiO_2-NPs ml^{-1} in RMPI-1640 culture medium for 24 h. The treated cells were centrifuged and processed for analysis using a JEOL JEM 2100 TEM (JEOL Ltd., Tokyo, Japan). Sample slices were mounted on carbon-coated copper grids and imaged. The chemical element composition was analyzed using an energy-dispersive X-ray spectroscopy (EDS) machine (EDAX Inc., Mahwah, NJ, USA); this analysis was performed simultaneously with the TEM imaging to evaluate the elemental composition of the material in the TEM images.

Ames Test

The tester strains used in the Ames test were *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102. A pre-incubation assay (Maron and Ames 1983) using 0, 38.4, 76.8, 153.6, 307.2, 614.4, 1228.8, 2457.6 and 4915.2 μg per plate of the TiO_2-NPs without S9 activation was conducted according to Organization for Economic Cooperation and Development (OECD) Guideline no. 471 (OECD 1987). For pre-incubation, the tester strains were incubated in nutrient broth II with TiO_2-NPs for 4 h at 37 °C with shaking at 80 rpm. Before incubation, the TiO_2-NPs were dispersed by vortexing for 5 min followed by 10 min of bath sonication in a Branson B2510 (Branson Ultrasonics, Danbury, CT, USA) with 100 W output power and 42 kHz frequency. Serial two-fold dilutions of TiO_2 NPs were used, beginning at 38.4 μg per plate and continuing up to 4915.2 μg per plate. The vehicle and an appropriate mutagenic chemical were used as the negative and positive controls for each individual tester strain. After 4h incubation, top agar was added to the treatment mixture, which was then poured on a minimal glucose agar plate. The plate was then incubated for 48 h at 37 °C. After the incubation, the plates were observed and the colonies formed were counted using a Quebec® Darkfield Colony Counter (Reichert Technologies, Depew, NY, USA).

TK6 Cell Culture and Treatments

The TK6 human lymphoblastoid cells and cell culture reagents were purchased from the American Type Culture Collection (Manassas, VA, USA). TK6 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of

Table 1. Genotoxicity testing of titanium dioxide nanoparticles in literature

Size and structure	Testing tissue or cellline	Testing system	Concentration	Exposure duration	Findings	Reference
25 nm; 70–85% anatase, 30–15% rutile	Human peripheral blood lymphocytes	Comet assay; Micronucleus assay	20, 50 and 100 $\mu g\ ml^{-1}$	6, 12, 24 h for the Comet assay; 20 h for the micronucleus assay	Significantly increased micronucleus formation and DNA breakage were observed.	(Kang *et al.*, 2008)
~15 nm	NIH 3 T3 cells and human fibroblast HFW cells	Anchorage-independent growth assay	10 $\mu g\ ml^{-1}$	1-12 week	Long-term exposure disturbed duplicated genome segregation, leading to chromosomal instability.	(Huang *et al.*, 2009)
40 -70 nm; Anatase	Human sperms and lymphocytes	Comet assay	3.73, 14.92, 29.85 and 59.7 $\mu g\ ml^{-1}$	30 min	DNA damage is induced both in dark and after pre-irradiation with UV or simultaneous irradiation with UV, with more DNA damage for the irradiation than that in dark.	(Gopalan *et al.*, 2009)
<100 nm	Human WIL2-NS cells	Micronucleus assay; Comet assay; *HPRT* mutation assay	0, 26, 65 and 130 $\mu g\ ml^{-1}$	6, 24 and 48 h	Approximately 2.5-fold increases in the frequency of micronucleated binucleated cells at 130 $\mu g\ ml^{-1}$; Approximately 5-fold increases in olive tail moment at 65 $\mu g\ ml^{-1}$; Approximately 2.5-fold increases in the mutant frequency in the *HPRT* gene at 130 $\mu g ml^{-1}$	(Wang *et al.*, 2007)
<100 nm; Anatase	Human diploid fibroblasts and bronchial epithelial cells	Comet assay	2, 5, 10, 50 $\mu g\ cm^{-2}$	24 h	Negative results	(Bhattacharya *et al.*, 2009)
21.9 nm; 77% anatase 25.2 nm; 90% anatase	Human colon adenocarcinoma cell line Caco-2	Fpg-Modified Comet Assay	20 $\mu g\ cm^{-2}$	4 h	Only anatase-rutile containing samples, in contrast to the pure anatase	(Gerloff *et al.*, 2009)

(Continues)

Table 1. (Continued)

Size and structure	Testing tissue or cellline	Testing system	Concentration	Exposure duration	Findings	Reference
6.7 nm; anatase 3.94 nm; anatase					samples, induced mild DNA damage	
21 nm, anatase 25.5 nm, anatase 25 nm, rutile 42 nm, rutile	*Salmonella typhimurium*; Mouse lymphoma L5178Y Cells; Chinese hamster cells;	Ames test; Comet assay; Mouse lymphoma assay; Chromosomal aberration assay	Various doses for the different size and form materials	Ames test: 48 h; Comet assay: 1 h; Mouse lymphoma assay: 1 h; Chromosomal aberration assay: 1 h	Negative or weak genotoxicity in all tests.	(Nakagawa *et al.*, 1997)
20 nm; Anatase with hydrophilic or hydrophobic surface	Female Wistar rats	Quantification of 8-oxoGua	0.15–1.2 mg by intratracheal instillation	Animals were euthanized 3, 21, or 90 days after instillation.	Negative results	(Rehn *et al.*, 2003)
100 nm; anatase 100 nm; rutile 100 nm; 79% rutile and 21% anatase	*Salmonella typhimurium* *Escherichia coli* Chinese hamster ovary cells	Ames test; *In vitro* mammalian Chromosome aberration test	Ames test: 100–5000 μg per plate; Chromosome aberration test: 750–2500 μg ml^{-1} for 4 h without S9; 62.5–250 μg ml^{-1} for 4 h with S9; 25–100 μg ml^{-1} for 20 h without S9	Ames test: 48 h; Chromosome aberration test: 4 h with or without S9 conditions and 20 h without S9	Negative in all the tests	(Warheit *et al.*, 2007)

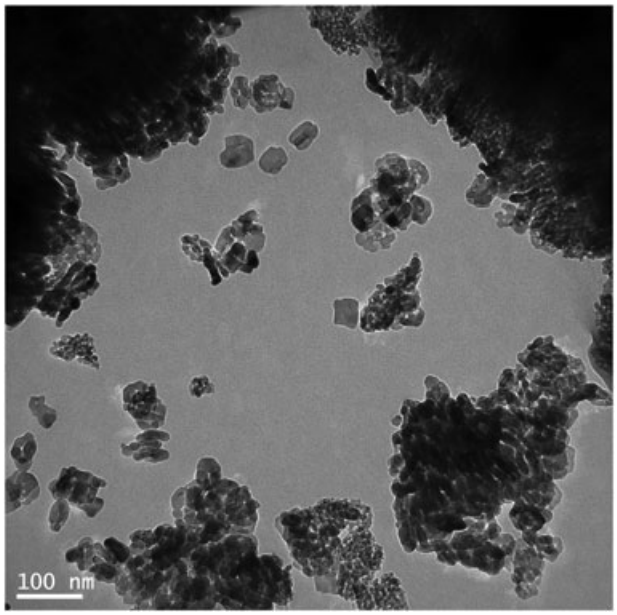

Figure 1. Representative transmission electron microscope (TEM) image of 10 nm titanium dioxide nanoparticles (TiO_2-NPs) powder. Magnification is × 50 K. The scale bar represents 100 nm.

5% CO_2. One day before the treatment, 3×10^5 cells were seeded into T-25 flasks containing 5 ml of growth medium and incubated overnight. The cells were then treated with 0, 50, 100, 150 and 200 μg ml^{-1} TiO_2-NPs in triplicate for 24 h with gentle shaking in the dark. The dispersion procedure for TiO_2-NPs was the same as that used for the Ames assay. Water and 20 μg ml^{-1} methyl methanesulphonate (MMS) served as the negative and positive controls, respectively.

Comet Assay

After treatment, cell viability was tested using Trypan blue dye exclusion. Next, 10 μl 0.4% trypan blue was added in 100 μl of cell culture, and a 10μl mixture of the trypan blue/cell was added on a hemacytometer (Thomas Scientific, Swedesboro, NJ, USA.) and the number of cells was determined under a Nikon TMS-F microscope (Nikon Corporation, Tokyo, Japan) at low magnification. Calculation of the total number of cells per ml culture is as previously described (Strober 2001): cells/ml = average count per square × dilution factor × 10^4. The dilution factor in the present study is 1.1.

A comet assay was performed with a kit purchased from Trevigen Inc. (Gaithersburg, MD, USA) and the assay was conducted according to the method described by the manufacturer's instruction manual. After the treatment, the cell solutions were pipetted to make single-cell suspensions and then the suspensions were combined with molten low melting point agarose at a ratio of 1:10 (v/v). Next, 50 μl of the solution was immediately pipetted onto glass slides. The slides were then placed in a dark refrigerator at 4 °C for 10–15 min. The slides were then immersed in a pre-chilled lysis solution and placed back in the refrigerator for at least 1 h. The lysis buffer was then removed and the slides were immersed in alkaline unwinding solution for 20 min in the dark at room temperature. The slides then were removed from the alkaline unwinding solution and electrophoresed at 0.7 V/cm for 30 min in the dark. After the electrophoresis, the slides were soaked three times in neutralizing buffer (0.4 M Tris, adjusted to pH 7.5 with HCl) for 5 min each and then immersed in 100% ethanol for 5 min. The slides were then allowed to dry before being stained with 100 μl of diluted SYBR Green for 5 min in a dark refrigerator. The slides were then analyzed using a system consisting of a Nikon 50i fluorescent microscope and Comet IV digital imaging software (Perceptive Instruments, Wiltshire, UK). The Comet tail lengths were digitally analyzed and scored based on tail length, width and intensity. Percent (%) DNA in the tail, defined as the fraction of DNA in the tail divided by the total amount of DNA associated with a cell multiplied by 100, was used as the parameter for DNA damage analysis using the software.

The endonuclease III (*Endo*III) modified Comet assay was performed as described previously (Ding *et al.*, 2011). The human 8-hydroxyguanine DNA-glycosylase (hOGG1)-modified Comet assay was performed in a similar way as *Endo*III, the hOGG1 was diluted by enzyme buffer by 1:1000 (v/v) and the incubation time at 37 °C was 30 min. Both enzymes were obtained from New England Biolabs (Ipswich, MA, USA).

Data Analysis and Statistics

For the Ames test, positive and negative responses were defined as described previously (Li *et al.*, 2011). When treatments caused a dose-dependent response and at least one treatment group induced two-fold or more change in the number of revertant colonies over the control, the testing agents were considered as positive. Negative response was defined as no dose-dependent increase in the number of revertant colonies. For the Comet assay, three slides were scored per sample; and 100 cells were randomly scored for each slide. A statistical significant difference ($P < 0.05$) between the treatment and the control group was considered a positive response.

Data for mean and standard deviation were generated from three independent experiments. All the data were analyzed for significance by one-way analysis of variance (ANOVA) using SigmaPlot version 11.0 (SPSS, Chicago, IL, USA). All analyzes were one-tailed, and $P < 0.05$ was used to identify statistically significant differences.

Results

Ames Test

Eight different doses of TiO_2-NPs (0–4915.2 μg per plate) were used for the Ames test. No significant changes from the negative controls were found for any of the TiO_2-NPs treatments, whereas the positive control chemicals for the five strains significantly increased the mutant frequencies over the negative controls. According to the OECD Guideline no. 471 (OECD 1987), a negative result should be confirmed using the highest concentration up to 5 mg per plate. The highest concentration in this study was 4915.2 μg ml^{-1} (i.e. about 5 mg per plate), and did not significantly induce any more colonies than the negative controls (Table 2). The positive and negative results are similar to our historical positive and negative data. Thus, the TiO_2-NPs were not genotoxic in the Ames test.

Table 2. Mutagenicity of 10 nm titanium dioxide nanoparticles (TiO_2-NPs) in *Salmonella typhimurium* tester strains

Dose (μg per plate)	Number of colonies per plate (mean ± SD, $n = 3$)				
	TA 1537	TA 98	TA 100	TA 102	TA 1535
0.0	9 ± 3	28 ± 26	184 ± 58	381 ± 67	20 ± 2
38.4	7 ± 3	36 ± 33	191 ± 33	383 ± 55	22 ± 7
76.8	8 ± 2	34 ± 30	157 ± 18	453 ± 111	19 ± 4
153.6	9 ± 1	24 ± 30	176. ± 17	376 ± 45	20 ± 8
307.2	14 ± 1	49 ± 39	160 ± 11	444 ± 85	20 ± 1
614.4	9 ± 4	35 ± 29	164 ± 23	352 ± 34	26 ± 4
1228.8	12 ± 3	28 ± 35	150 ± 35	376 ± 42	18 ± 4
2457.6	11 ± 4	48 ± 37	155 ± 12	384 ± 49	23 ± 13
4915.2	21 ± 9	42 ± 35	139 ± 19	364 ± 21	30 ± 16
Positive control	922 ± 76[a,***]	465 ± 27[b,***]	2604 ± 356[c,***]	777 ± 10[d,*]	847 ± 50[e,***]

Note: Three independent assays were performed and SD represents standard deviation. The chemicals for the positive controls are [a]ICR-191 (1 μg per plate), [b]2-nitrofluorene (3 μg per plate), [c]nitrofurantoin (5 μg per plate), [d]mitomycin (0.2 μg per plate) and [e]sodium azide (1 μg per plate). * and *** indicates $P < 0.05$ and < 0.01 vs. control.

Comet Assay

Cell viability in TK6 cells treated with TiO_2-NPs was evaluated using the Trypan blue dye exclusion assay. As shown in Fig. 2A, TiO_2-NPs treatment caused cytotoxicity in TK6 cells in a concentration-dependent manner. At the concentration of 200 μg ml^{-1}, the cell viability was around 55%. In the Comet assay, treatment of 50, 100, 150 and 200 μg ml^{-1} TiO_2-NPs did not increase the DNA damage in either the standard assay or the enzyme-modified assay, suggesting that no DNA breakage or oxidative DNA adducts were caused by the treatment. The positive control (MMS) significantly caused induction of DNA breaks and oxidative DNA adducts in the Comet assay except in the hOOG1-modified Comet assay because the hOOG1 enzyme does not recognize alkylating damage induced by MMS (Smith *et al*., 2006) (Fig. 2B).

Uptake Analysis of TiO_2-NPs by Cells

To assure that the negative results from the Ames test and Comet assay were not due to a lack of exposure, TiO_2-NPs uptake by the bacteria and TK6 cells was examined. After incubation of *S. typhimurium* (TA100) and TK6 cells with

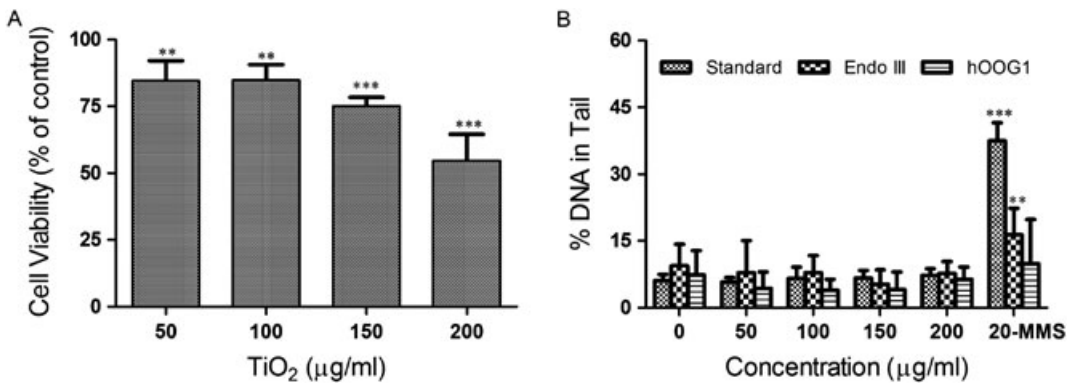

Figure 2. Effects of 10 nm titanium dioxide nanoparticles (TiO_2-NPs) on DNA damage evaluated with the Comet assay. After treatment of TK6 cells with TiO_2-NPs (0–200 μg ml^{-1}) for 24 h, the cells were collected and the slides were prepared as described in the Materials and Methods. DNA damage was measured as % DNA in the tail. (A) Effects of TiO_2-NPs on cell viability. The analysis was conducted with the Trypan blue dye exclusion assay. ** and ***indicate $P < 0.01$ and $P < 0.001$ vs. the buffer control, respectively. (B) Effects of TiO_2-NPs on DNA damage in the standard Comet assay and endonucleases enzyme-modified Comet assay. In all, 20 μg ml^{-1} methyl methanesulphonate (MMS) was used as the positive control. Endonuclease III (*Endo*III) and hOOG1 were added in the enzyme-modified Comet assay. Three independent experiments were performed and the error bars represent the standard errors of the mean. None of the TiO_2-NPs treatments increased DNA damage in either of the assays.

200 mg μl^{-1} TiO_2-NPs for 4 and 24 h, respectively, the cells were sampled using TEM and elemental analyzes. More than 20 images from each treatment, along with their control samples, were analyzed. As shown in a representative TEM image for the control TK6 cells, no TiO_2-NPs were found (Fig. 3A) and no elemental titanium was detected inside the cells on any of the TEM images (Fig. 3B). In contrast, TiO_2-NPs were found in nearly every TEM image prepared with the TK6 cells treated with the TiO_2-NPs (Fig. 3C, D). Figure 3C shows the nanoparticles inside of a treated cell and Fig. 3D confirms that the particles are TiO_2-NPs because the main chemical composing the particles is titanium. On the other hand, TiO_2-NPs were not found inside the control or the treated bacteria in any of the samples imaged although aggregated TiO_2-NPs were frequently found outside the treated bacteria (Fig. 4A–C). Elemental titanium was rarely found by EDS analysis within the bacterial cells from treated or untreated bacterial samples (Fig. 4B, F), whereas existence of TiO_2-NPs outside of the bacteria was confirmed by EDS analysis (Fig. 4D). It should be noted that the high peaks for carbon (C), copper (Cu) and lead (Pb) in Fig. 4B, D and F mainly came from the cells and TEM grids.

Discussion

In all, 10 nm TiO_2-NPs had no mutagenetic ability in any of the five bacterial tester strains, even at a concentration of 4915.2 μg per plate, which is close to the highest concentration (5mg per plate) recommended by OECD Guideline no. 471 (OECD 1987) for a negative result. Our results are consistent with many others that demonstrate nanoparticles generally are negative in the Ames test (Dufour *et al.*, 2006; Kisin *et al.*, 2007; Li *et al.*, 2011; Maenosono *et al.*, 2007; Nakagawa *et al.*, 1997; Warheit *et al.*, 2007). Several mechanisms have been suggested for the negative results of nanomaterials in Ames test.

One of these hypotheses is that nanoparticles do not penetrate the cell wall of bacterial cells, suggesting that the Ames test is unsuitable for evaluating the genotoxicity of nanoparticles (Landsiedel *et al.*, 2009; Nabeshi *et al.*, 2011; Singh *et al.*, 2009). However, Kumar *et al.* (2011) reported a positive result for Ames tests conducted for ZnO-NPs and TiO_2-NPs and claimed that unagglomerated nanoparticles were observed inside the treated bacterial cells. However, their results for the internalization of the nanoparticles are questionable. The nanoparticles inside the bacterial cells are much smaller than those indicated by the article. The authors reported that the average size of ZnO-NPs and TiO_2-NPs observed by TEM were 30 and 50 nm, respectively, but the sizes of the nanoparticles shown in the TEM images are clearly smaller than 5 nm. In addition, no chemical component analysis of the nanoparticles inside the bacteria was conducted. Thus, it was unclear whether the black points indicated by the arrows in the figures are nanoparticles or something else. Similar black dots were also found in our TEM images and they were confirmed not to be nanoparticles (Fig. 4A). In the present study, more than 20 TEM images and chemical

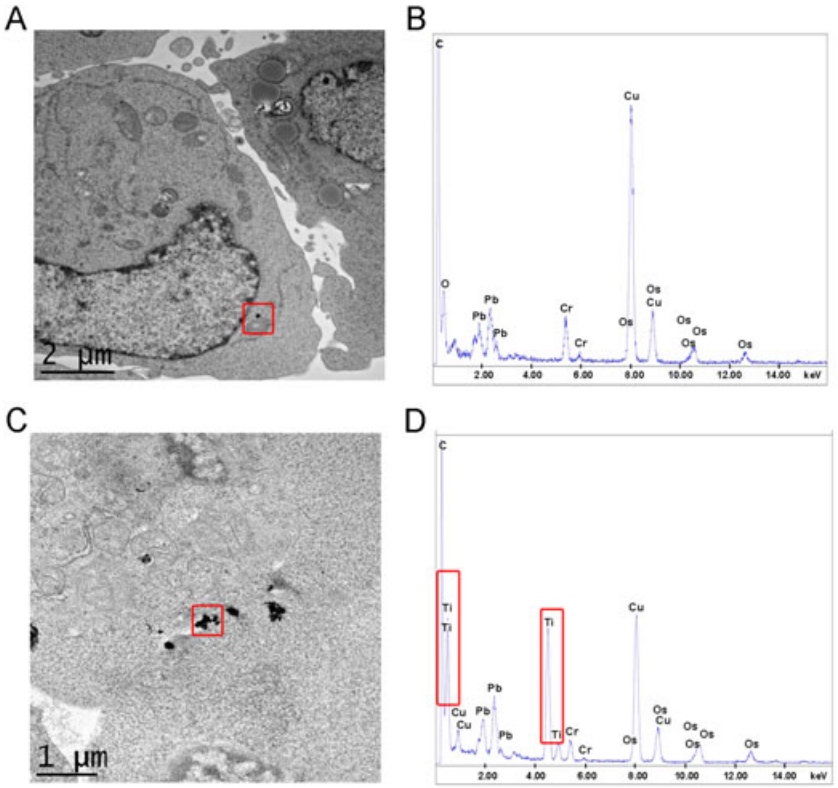

Figure 3. Cellular uptake of titanium dioxide nanoparticles (TiO_2-NPs) in TK6 cells. (A) A transmission electron microscope (TEM) image of a part of a TK6 cell treated with the vehicle control. The scale shown in the graph is 2 μM. (B) Chemical composition analysis of an area inside the cell in (A) using energy-dispersive X-ray spectroscopy (EDS). (C) A TEM image of a partial TK6 cell treated with 200 μg ml^{-1} TiO_2-NPs. The scale in the graph is 1 μM. (D) Chemical composition analysis of an area of the cell in Graph C by EDS. The areas that were analyzed by EDS on the TEM images are indicated by the red boxes. The red boxes in (D) indicate the titanium element.

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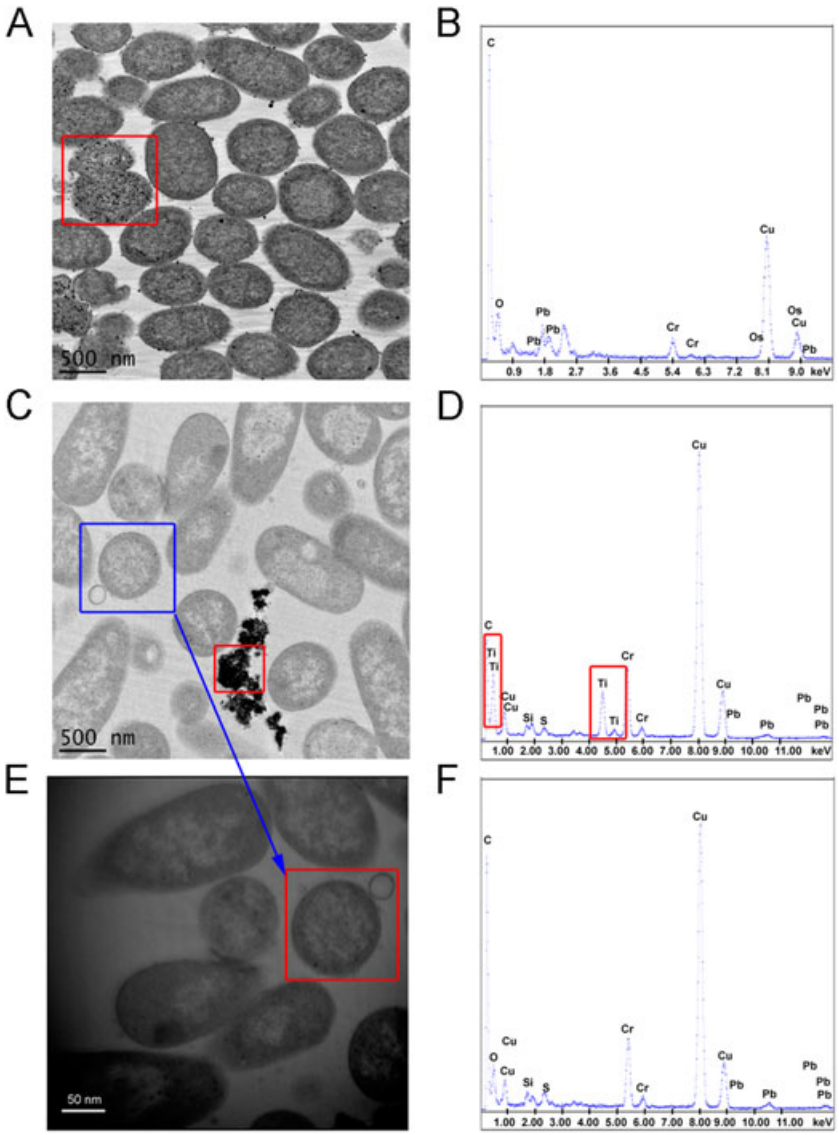

Figure 4. Uptake of the titanium dioxide nanoparticles (TiO_2-NPs) in *Salmonella* cells. Transmission electron microscope (TEM) and chemical composition analyses were applied to examine the uptake of TiO_2-NPs in *Salmonella* TA 100 cells. (A) A representative TEM image of the bacterial cells treated with the vehicle control. (B) Chemical composition analysis of the bacteria in (A) by energy-dispersive X-ray spectroscopy (EDS) showing no titanium existence. (C) A representative TEM image of the bacterial cells treated with 200 μg ml^{-1} TiO_2-NPs. (D) Chemical composition analysis of TiO_2-NPs agglomerates outside of the bacterial cells by EDS. (E) High resolution TEM image of a bacterial cell in (C). (F) Chemical composition analysis of the bacterial cell by EDS. The scales are 500 nm for (A) and (C) and 50 nm for (E). The areas that were analyzed by EDS in (A), (C), (E) are indicated by the red boxes. The red boxes in (D) show the titanium element.

composition assays by EDS failed to identify any TiO_2-NPs within *S. typhimurium* tester cells (Fig. 4). To the best of our knowledge, this is the first direct evidence based on both TEM and EDS that 10 nm TiO_2-NPs cannot enter bacterial cells probably because prokaryotes cannot uptake the nanoparticles as a result of the absence of endocytosis; thus the nanoparticles that cannot enter bacterial cells will not be able to damage DNA and induce mutations (Doak *et al.*, 2012). The result strongly supports the hypothesis that nanomaterials may not cross the bacterial wall and the Ames test might not be a suitable assay for assessing genotoxicity of nanoparticles (Landsiedel *et al.*, 2009).

The *in vitro* Comet assay is a sensitive method for evaluating DNA damage. The standard assay measures DNA strand breaks whereas the enzyme-modified Comet assay detects oxidative DNA adducts. *Endo*III and hOGG1 applied in this study recognize oxidized pyrimidines including thymine glycol and uracil glycol (Collins *et al.*, 1993; Dizdaroglu 2005) and 8-oxo-7,8-dihydroguanine (8-oxoGua) (Radicella *et al.*, 1997; Rosenquist *et al.*, 1997), respectively. Although uptake of TiO_2-NPs by TK6 cells clearly occurred (Fig. 3), there was neither a significant induction of DNA breaks in the standard Comet assay nor oxidative DNA adducts in the enzyme-modified Comet assay (Fig. 2B). Similar negative results from Comet assay testing of TiO_2-NPs exposed to human diploid fibroblasts, human bronchial epithelial cells, human carcinoma intestinal cells and human keratinocytes were reported (Bhattacharya *et al.*, 2009; Gerloff *et al.*, 2009; Serpone *et al.*, 2006). However, other researches have shown that TiO_2-NPs were positive in the Comet assay (Dunford *et al.*, 1997; Gopalan *et al.*, 2009; Gurr *et al.*, 2005;

Karlsson *et al*., 2008; Reeves *et al*., 2008; Vevers and Jha 2008; Wang *et al*., 2007). The inconsistent findings may result from different sizes and structure of TiO_2-NPs used in the studies. Also the lack of physicochemical characterization information in some reports makes it difficult to compare the results among studies.

TiO_2-NPs are considered safe for use in sunscreens at the intended use level by the US Food and Drug Administration, but there is considerable concern with photogenotoxicity of TiO_2-NPs (Bhattacharya *et al*., 2009; Dunford *et al*., 1997; Gerloff *et al*., 2009; Gopalan *et al*., 2009; Gurr *et al*., 2005). In a preliminary study, we treated TK6 cells with 200 μg ml^{-1} TiO_2-NPs for 4 h and conducted the Comet assay both in the dark and under ambient light, and only found a concentration-dependent induction of DNA damage in the light condition and no DNA damage was detected in the dark condition (data not shown). Similar negative results were also found in the present study with 24-h treatment of TiO_2-NP samples that were kept in dark as much as possible during the whole Comet assay. This is important as the DNA strand breaks can be significantly higher even when preparing the slides under normal laboratory lighting compared with slides prepared in the dark (Gerloff *et al*., 2009). The different results from the different experimental conditions may contribute to the inconsistent Comet assay results. Thus, it is necessary to pay close attention to light exposure while utilizing the Comet assay to assess genotoxicity of TiO_2-NPs and other nanoparticles in order to avoid false-positive effects.

In conclusion, 10nm TiO_2-NPs were not genotoxic in either the Ames test or Comet assay. Under the conditions we used for the Comet assay, TiO_2-NPs did not induce DNA strand breaks or oxidative adducts. The Ames test results were negative because TiO_2-NPs did not enter the tester bacterial cells. Thus, the Ames test might not be suitable for detecting the genotoxicity of nanoparticles.

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