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Pre-irradiation of anatase TiO_2 particles with UV enhances their cytotoxic and genotoxic potential in human hepatoma HepG2 cells

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ABSTRACT

Titanium dioxide (TiO_2) is active in the UV region of the light spectra and is used as a photocatalyst in numerous applications. Photo-activated anatase TiO_2 particles promote increased production of free radicals. This is a desirable property, although the potential toxicity of such photo-activated TiO_2 particles on exposure of humans and the environment remains unknown. Therefore, we studied whether pre-irradiation of TiO_2 particles with UV influences their cytotoxic and genotoxic potential. The TiO_2 particles, as TiO_2-A (<25 nm) and TiO_2-B (>100 nm), were UV pre-irradiated (24 h) and tested for cytotoxic and genotoxic activities in human hepatoma HepG2 cells. Non-irradiated TiO_2-A/B at 1.0–250 μg/ml did not reduce viability of HepG2 cells, nor induce significant increases in DNA strand breaks; only TiO_2-A induced significant increases in oxidative DNA damage. After UV pre-irradiation, both TiO_2-A and TiO_2-B reduced cell viability and induced significant increases in DNA strand breaks and oxidative DNA damage. This is the first study that shows that UV pre-irradiation of anatase TiO_2 particles results in increased cytotoxic and genotoxic potential. This warrants further studies as it has important implications for environmental and human health risk assessment and preventive actions to limit human exposure.

1. Introduction

Titanium dioxide (TiO_2) is widely used as a white pigment in the production of paints, paper, ceramics, and as a food additive [1–4]. It is inexpensive, relatively stable chemically, and strongly absorbs UV light. TiO_2 is believed to be safe for use: in early studies, submicron-sized TiO_2 particles (>100 nm) were classified as harmless [5,6]. However, safe use of TiO_2 nanoparticles (NPs) is questionable, as there have been reports from the literature of potential toxicity, with oxidative stress as the main toxicity mechanism [4,7–11].

Under UV light irradiation the physical properties of TiO_2 change, as UV activates its photocatalysis. TiO_2 is the most investigated photocatalyst system and it has been shown to promote the decomposition of a variety of organic and inorganic compounds, which implies potential applications in sterilisation, sanitation, and pollution remediation [12,13]. Materials coated with TiO_2 are already in use, and they show self-cleaning, anti-fogging, and antibacterial properties [14].

The basic principle of semiconductor photocatalysis involves photon-generated electrons (e^-) and holes (h^+) that migrate to the surface and serve as redox sources that react with adsorbed reactants, which leads to the destruction of pollutants [15]. The photocatalytical properties of TiO_2 depend on the crystalline structure, particle size and specific surface area. Since the anatase crystalline structure has the highest photocatalytic activity, it is the most commonly used form of TiO_2 for such purposes [12,13]. Particle size is also an important parameter for catalysis in general, as it directly impacts on the specific surface area of a catalyst. With smaller particle sizes, the numbers of active surface sites increase, as does the surface charge-carrier-transfer rate in photocatalysis [15]. Due to this, TiO_2 NPs represent better photocatalyst material than larger, submicron-sized TiO_2 particles, so TiO_2 NPs are increasingly used nowadays instead of larger TiO_2 particles [2,3]. As photocatalytic activation changes the properties of TiO_2 towards a greater generation of reactive oxygen species (ROS), it is likely that this will also change its toxicity potential. Many studies have shown that TiO_2 is toxic only in the presence of UV irradiation [16,17], and that in the presence of UV irradiation the toxicity of TiO_2 is higher than in the dark [18–21]. However, in all of the studies published to date, cells were simultaneously exposed to UV irradiation and TiO_2, and therefore toxic or genotoxic effects of the UV irradiation alone cannot be excluded. Indeed, it is well known that UV irradiation is genotoxic through direct photochemical reactions with DNA, while also

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having indirect genotoxic effects that occur through the production of ROS by photosensitisation of biological molecules other than DNA [22,23], which can all lead to cell mutations or cell death [24].

To exclude the cytotoxic and genotoxic effects of UV irradiation *per se*, we compared the cytotoxic and genotoxic potential of non-irradiated and UV pre-irradiated anatase TiO_2 particles in an experimental model of human hepatoma HepG2 cells. We used two sizes of anatase TiO_2 particles, as TiO_2-A (<25 nm) and TiO_2-B (>100 nm). The effects of non-irradiated and UV pre-irradiated TiO_2 particles on cell viability were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Induction of DNA damage was determined with a comet assay, and induction of oxidative DNA damage with a modified version of the comet assay with the lesion-specific DNA-repair enzyme formamidopyrimidine-DNA glycosylase (Fpg), which converts oxidised purines to apurinic sites and strand breaks [25].

2. Materials and methods

2.1. Chemicals

MTT, *tert*-butyl hydroperoxide, benzo(a)pyrene, and dimethyl sulphoxide were all obtained from Sigma–Aldrich (USA). The Fpg enzyme was a gift from Dr. Andrew R. Collins (Department of Nutrition, University of Oslo, Oslo, Norway).

2.2. Characteristics of TiO_2 nanoparticles

We used two commercial anatase TiO_2 particles from Sigma–Aldrich (USA), which we abbreviate as TiO_2-A (Cat. No. 637254: anatase; particle size <25 nm; surface area 200–220 m^2/g) and TiO_2-B (Cat. No. T8141: anatase; no data provided about size and specific surface area). Their sizes, specific surface areas, zeta-potentials and phase compositions were determined experimentally.

The sizes and morphologies of TiO_2 particles were examined by field-emission-gun scanning electron microscopy (FEG-SEM) with a JEOL 7600 F instrument, and transmission electron microscopy (TEM) with JEM 2010F and JEOL instruments. The phase composition of the particles was verified by X-ray diffraction (Bruker AXS D4 Endeavor) using CuKα radiation, and the average crystallite size was calculated according to the Scherrer formula [26]. The specific surface areas were determined by gas adsorption using the BET method (Gemini 2370, Micromeritics). Zeta-potentials were measured before and after UV irradiation, in 3 wt.% suspensions in distilled water and in cell-growth medium, by the electrokinetic sonic amplitude (ESA) technique, using a ZetaProbe device (Colloidal Dynamics, USA).

2.3. TiO_2 particles, stock-solution and treatment-media preparation

The UV pre-irradiation of the TiO_2-A and TiO_2-B particles (dry, without mixing) was performed by 24 h irradiation in a UV chamber (I-265 CK UV, Kambič Laboratory Equipment) as a simulated sun spectrum with Osram UV bulbs without UVC (ULTRA VITALUX, 300 W, wavelength >290 nm). The stock suspensions were prepared immediately after the end of the UV irradiation of TiO_2 particles (within 15 min maximum). Afterwards, the procedure was the same for the non-irradiated and UV-irradiated TiO_2 particles. For both non-irradiated and UV-irradiated TiO_2 particles, the stock suspensions were prepared at 10 mg/ml in PBS. These were sonicated for 30 min in an ultrasonic bath (Sonorex, Bandelin Electronic, Germany) at a frequency of 60 kHz, to ensure uniform suspension. These stock suspensions were subsequently diluted in cell-growth medium, to final concentrations from 1 μg/ml to 250 μg/ml. These samples were then sonicated for 30 min, to produce non-agglomerated suspensions, before addition to the cells in culture. The time from the end of the UV irradiation and the cell treatment was 1 h maximum.

During the experimental work, illumination of the particles was avoided as much as possible (using aluminium foil to wrap the tubes); however, the experiments were not conducted in complete darkness. During the exposure of the cells to the TiO_2-A and TiO_2-B, the incubations were kept in complete darkness in a 5% CO_2 incubator at 37 °C.

2.4. Cell culture

The HepG2 cells were obtained from the European Collection of Cell Cultures (UK) and they were grown as described previously [10].

2.5. Determining cytotoxicity: the MTT assay

Cytotoxicity was determined with the MTT assay according to Mosmann [27], with minor modifications [28]. This assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of intact mitochondria of living cells. The HepG2 cells were seeded into 96-well microplates (Nunc, Naperville, USA) at a density of 40,000 cells/ml, and incubated for 20 h at 37 °C for their attachment. The cell-growth medium was then replaced by fresh medium containing 0, 1, 10, 100 or 250 μg/ml TiO_2-A or TiO_2-B, and incubated for 4 h, 24 h and 48 h. Each experiment included negative control (cell-growth medium) and positive control (5 μM $CdCl_2$). The protocol was continued as described previously [28]. Cell survival was determined by comparing the optical density of the wells containing the TiO_2-A/TiO_2-B treated cells with those of the negative control. Five replicates per concentration and three independent experiments were performed. Student *t*-tests were used to analyse the differences between treated and control cells; $p < 0.05$ was considered as statistically significant.

2.6. Determining genotoxicity: the classical and modified comet assays

The HepG2 cells were seeded at a density of ca. 60,000 cells/ml into 12-well microtitre plates (Corning Costar Corporation, USA). After incubation at 37 °C in 5% CO_2 for 20 h to allow the cells to attach, the cell-growth medium was replaced with fresh medium containing 0, 1, 10, 100 or 250 μg/ml TiO_2-A or TiO_2-B, and further incubated for 2 h, 4 h and 24 h. Each experiment included negative control (cell-growth medium) and positive controls (0.3 mM *tert*-butyl hydroperoxide and 50 μM benzo(a)pyrene). At the end of this treatment, the cells were harvested and DNA damage was determined by the protocol of Singh et al. [29], with minor modifications [30]. The level of oxidised purines was determined with a modified comet assay, as described by Collins et al. [25], with minor modifications [30]. Three independent experiments were performed for each of the treatment conditions. Percentages of tail DNA were used to measure the levels of DNA damage. One-way analysis of variance (ANOVA, Kruskal–Wallis) was used to analyse the differences between the treatments within each experiment. Dunnet's tests were used for comparing median values of percentage tail DNA; $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Characterisation of TiO_2-A and TiO_2-B

The general characteristics of the particles examined are summarised in Table 1, and their morphology is illustrated in Fig. 1. It

Table 1
Characteristics of the TiO_2 particles used in this study.

	TiO_2-A	TiO_2-B
Supplier information (Sigma–Aldrich)	Nanoparticle, anatase crystalline structure, particle size <25 nm	Particle, anatase crystalline structure
Analysis of particles, as-received (technique used, units)		
Specific surface area (BET, m^2/g)	129.3	8.6
Crystallite size within the agglomerates/aggregates (XRD, nm)	18	105
Agglomerates/aggregates size (FEG-SEM, μm)	~1	~50
Particle shape (TEM)	Elongated crystallites	Spherical crystallites
Crystal structure (XRD)	Anatase	Anatase

is evident from Fig. 1A, C, and E that TiO_2-A is composed of smaller, but more aggregated (firmly compacted), primary particles (crystallites), compared to TiO_2-B (Fig. 1B, D, and F). As illustrated in the TEM in Fig. 1E and F, the crystallites of TiO_2-A are elongated, while crystallites of TiO_2-B, are spherical. The TiO_2-B particles appear granulated, i.e. compacted into ca. 50 μm soft agglomerates (Fig. 1B), which prevents the crystallites from free flowing. Due to the larger size of the TiO_2-B crystallites, the specific surface area of TiO_2-B is significantly lower than that of TiO_2-A. However, as the specific surface area is measured in gas, it does not necessarily reflect the properties in liquid. In suspension, the granules of the TiO_2-B particles de-agglomerate and disperse, which significantly affects its colloidal behaviour. In both cases, X-ray diffraction confirms the anatase crystal structure (Fig. 2), although for the TiO_2-A particles the peaks are broadened due to the very small crystallite size. The average crystallite sizes calculated according to the Scherrer formula are 18 nm for TiO_2-A and 105 nm for TiO_2-B.

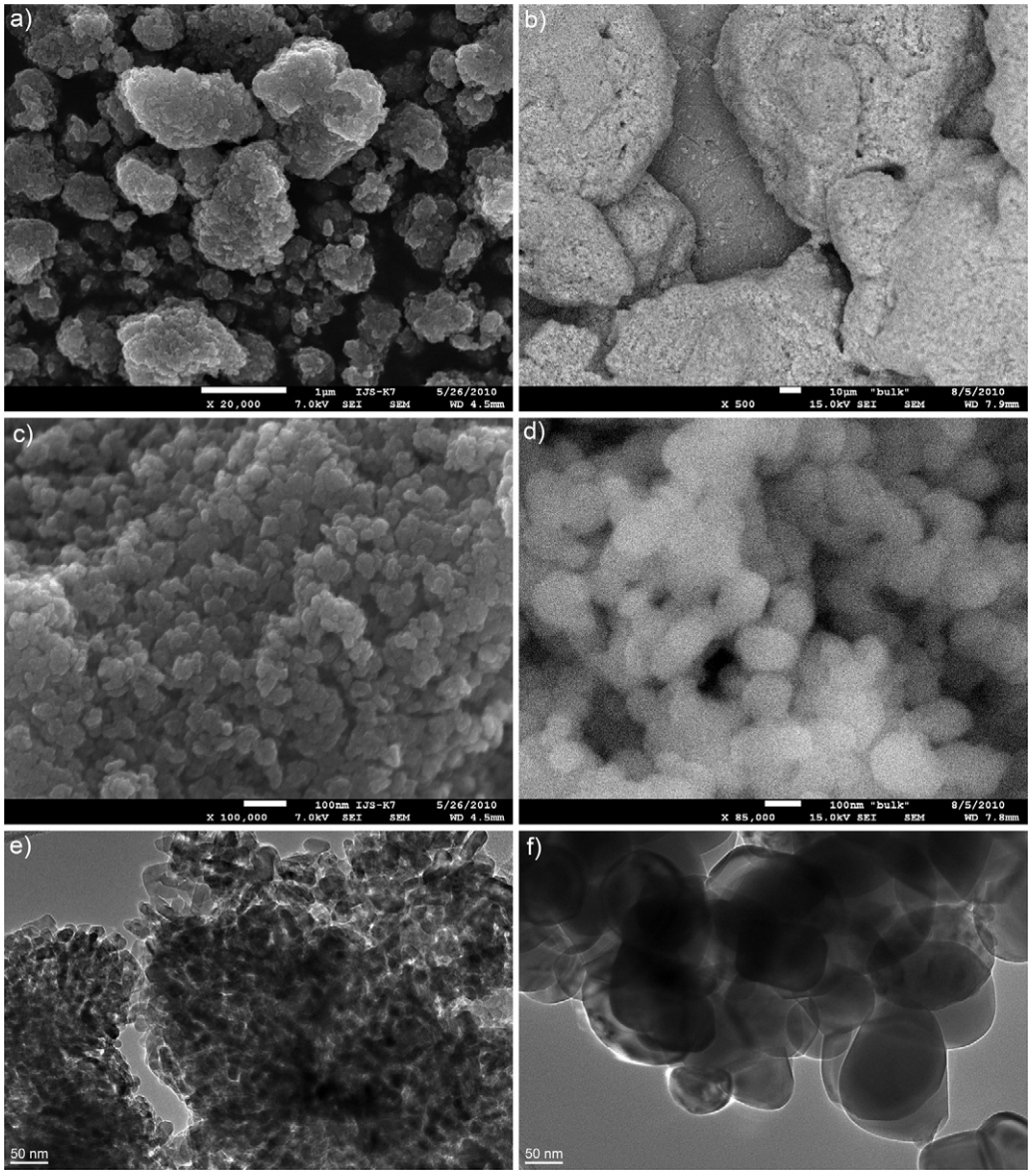

Fig. 1. FEG-SEM images of the TiO_2-A (A and C) and TiO_2-B (B and D) particles, and corresponding TEM images (E and F, respectively) (note: low magnification in B).

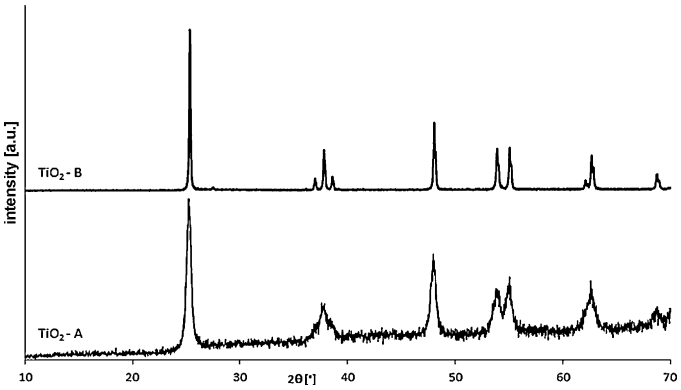

Fig. 2. X-ray diffractogram of the TiO_2-A and TiO_2-B particles (all of the peaks correspond to anatase TiO_2).

Table 2
The properties of the as-received TiO_2 particles in suspensions (3 wt.% solids).

Property, as-prepared	TiO_2-A	TiO_2-B
pH of aqueous suspension	6.5	7.8
Zeta-potential in water at pH 7 (mV)	−20	−98
Electrical conductivity (mS/cm)	0.20	0.10
Iso-electric point in water	6.9	1.4
Zeta-potential in medium (mV)	−8.7	−13.5
Stability of aqueous suspension	Fast sedimentation	Stable for days

As stated above, in addition to the characteristics of the particles as-received, their behaviours in liquids are also highly relevant. As given in Table 2, the particles examined revealed significantly different behaviours when mixed into water. The first observation is the difference in the pH of the suspensions at 3 wt.% solids: the natural pH of TiO_2-A was 6.5, while for TiO_2-B it was 7.8, which indicates different chemical compositions of the surface layers of the particles. This is also reflected in the large differences in electrical conductivities and iso-electric points, and in particular in the absolute values of the zeta-potentials at pH 7; the absolute values of the zeta-potentials were higher for TiO_2-B (−98 mV) than for TiO_2-A (−20 mV), which reflected the higher stability of the TiO_2-B suspensions than those of TiO_2-A. It is worth noting that the electrical conductivities increased and the zeta-potentials decreased with time, which indicates a degree of solubility of the particles.

As illustrated in Fig. 3, the highly negative surface charge of TiO_2-B in aqueous suspension appears within a wide pH range. In the range of physiological pH values, the TiO_2-B particles are consequently well dispersed and the suspension is stable,

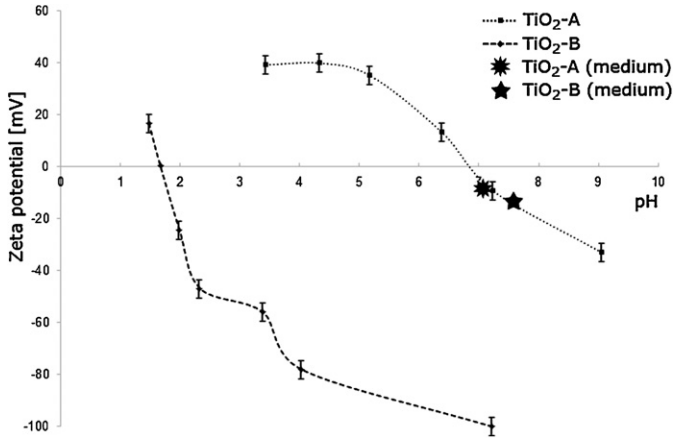

Fig. 3. Zeta potential of the TiO_2-A and TiO_2-B particles in water and in cell-growth medium (as indicated), as a function of pH.

while conversely, TiO_2-A flocculates around pH 7 (near its iso-electric point), which explains the observed poor dispersibility and fast sedimentation of TiO_2-A. It is also evident, that TiO_2-A is positively charged under acidic conditions. The opposite surface charges of these particles imply a tendency for binding to different biomolecules. This difference for TiO_2 particles with regard to their surface charges was also reported by Liao et al. [31].

The wide differences in zeta potentials for the TiO_2-A and TiO_2-B particles in aqueous suspensions are largely lost in cell-growth medium (Fig. 3). The absolute zeta potential value in cell-growth medium is −8.7 mV and −13.5 mV for TiO_2-A and TiO_2-B, respectively. This will be due to the high ionic strength of the cell-growth medium, as a consequence of the high concentrations of proteins and electrolytes.

We also determined whether the properties of the particles change during the 24 h of UV irradiation; here, the changes were insignificant and the morphology remained unchanged. The only change noted was the change in the conductivity and zeta potential of the suspension of the TiO_2-A particles: their conductivity decreased from 0.19 mS/cm before irradiation, to 0.135 mS/cm after irradiation, and their zeta potential increased from 19 mV to 25 mV. These findings suggest the presence, and the UV-induced degradation, of organic molecules on the surfaces of the particles as-received, which affect the behaviour of the suspensions.

Hence, although both of these particle types have the same chemistry (TiO_2) and crystal structures (anatase), they appeared different not only in crystallite size, but also in other chemical and physical properties that probably arise from their different synthetic routes. This can, in turn, affect their bioavailability and toxicity.

3.2. MTT assay

Survival of the HepG2 cells treated with non-irradiated and UV pre-irradiated TiO_2-A and TiO_2-B was determined using the MTT assay. The HepG2 cells were exposed to 0, 1, 10, 100 and 250 μg/ml non-irradiated or UV pre-irradiated TiO_2-A and TiO_2-B for 4 h, 24 h and 48 h in the dark.

Exposure of the HepG2 cells to non-irradiated TiO_2-A and TiO_2-B for up to 48 h did not affect their viability (Fig. 4). On the contrary, UV pre-irradiated TiO_2-A and TiO_2-B significantly ($p < 0.05$) decreased the viability of the HepG2 cells at the highest two doses (100 and 250 μg/ml), which was already evident after 4 h exposure to UV pre-irradiated TiO_2-A and TiO_2-B, and became even more pronounced during the prolonged exposure for 48 h to TiO_2-A (Fig. 4A–C) and TiO_2-B (Fig. 4D–F).

3.3. Induction of DNA strand breaks and oxidative DNA damage: classical and modified comet assays

The extent of DNA strand breaks after exposure of the HepG2 cells to non-irradiated and UV pre-irradiated TiO_2-A and TiO_2-B for 2 h, 4 h and 24 h was determined using the classical comet assay.

Exposure of the HepG2 cells to non-irradiated TiO_2-A induced a significant ($p < 0.05$) increase in DNA strand breaks at 250 μg/ml after 2 h, 4 h and 24 h (Fig. 5A–C). Exposure to UV pre-irradiated TiO_2-A for 2 h resulted in a significant ($p < 0.05$) increase in the level of DNA strand breaks at concentrations above 10 μg/ml (Fig. 5A), although after 4 h exposure, the increase was significant ($p < 0.05$) only at 250 μg/ml (Fig. 5B), while after 24 h exposure, significant ($p < 0.05$) increases were seen for both 100 μg/ml and 250 μg/ml (Fig. 5C).

In cells exposed to non-irradiated TiO_2-B for 2 h, the levels of DNA strand breaks were not significantly increased (Fig. 5D); however, with the longer exposure of 4 h and 24 h, there was a significant ($p < 0.05$) increase in DNA strand breaks compared to the

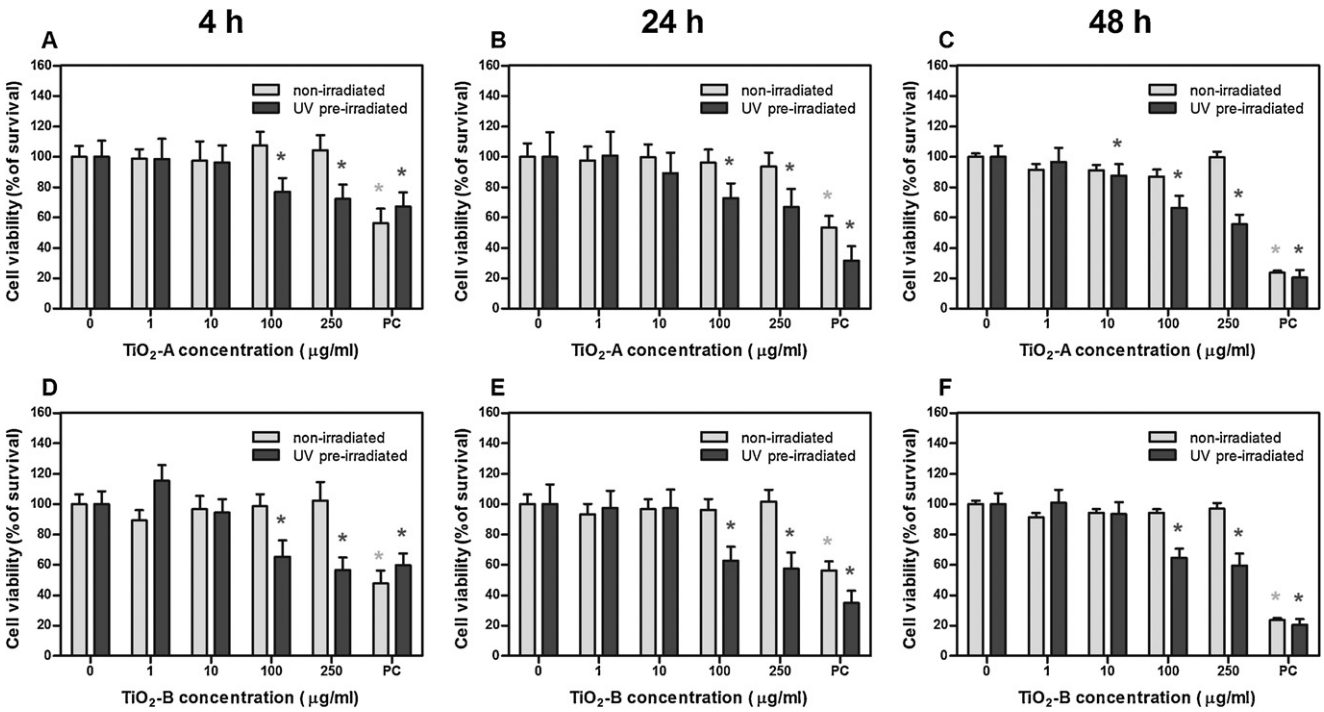

Fig. 4. The effects of non-irradiated and UV pre-irradiated TiO_2-A and TiO_2-B on the viability of HepG2 cells, as indicated: after 4 h (A and D), 24 h (B and E) and 48 h (C and F). Cell viability was measured using the MTT assay, as described in Section 2. PC, positive control of 5 μM $CdCl_2$ treatment. Data are means ±SD from three independent experiments (each with five replicates). $^*p < 0.05$, treated *versus* control cells (Student *t*-test).

relevant controls (Fig. 5E and F). With the exposure of the cells to UV pre-irradiated TiO_2-B, there were significant ($p < 0.05$) dose-dependent increases in the level of DNA strand breaks at 100 μg/ml and 250 μg/ml at all times of exposure (Fig. 5D–F).

The induction of oxidative DNA damage was studied with a modified comet assay with the purified Fpg enzyme (see Section 2) that recognises and excises oxidised purines, which are indicative of DNA strand breaks (Fpg-sensitive sites).

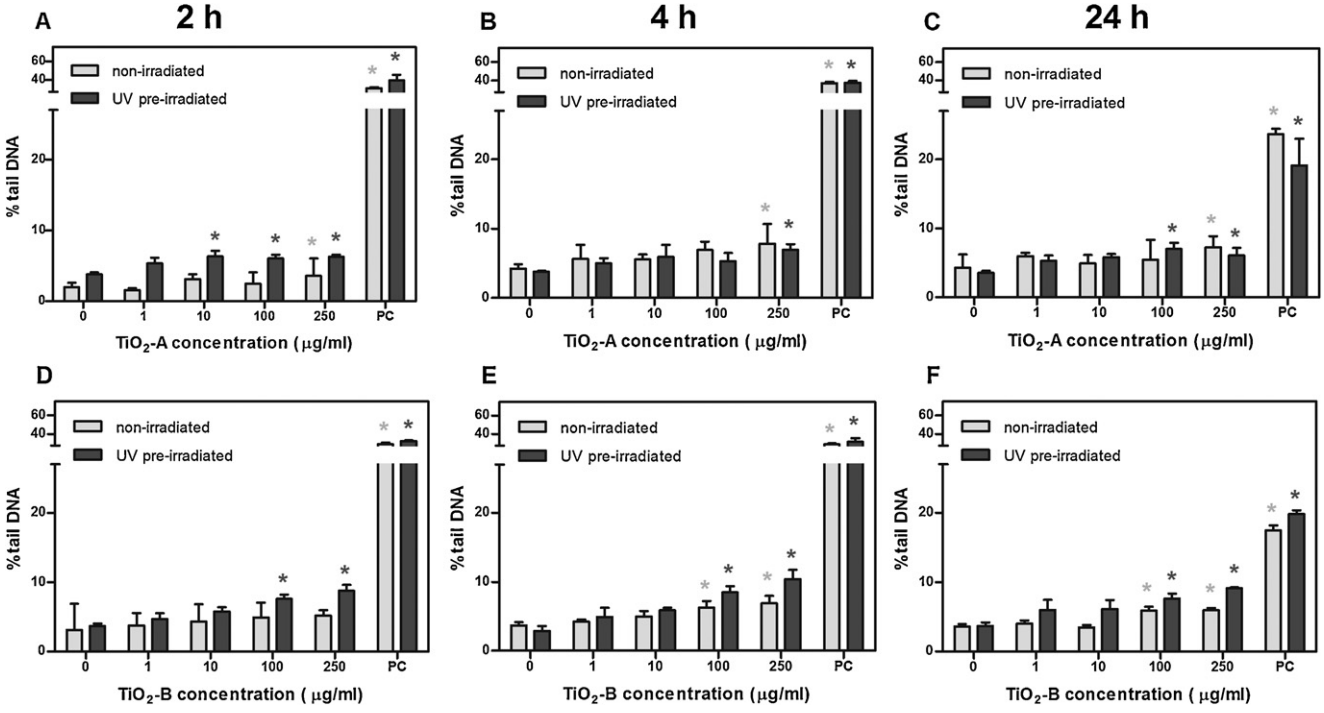

Fig. 5. The effects of non-irradiated and UV pre-irradiated TiO_2-A and TiO_2-B on induction of DNA strand breaks in HepG2 cells, as indicated: after 2 h (A and D), 4 h (B and D) and 24 h (C and F). DNA damage was assessed using the comet assay, as described in Section 2. PC, positive control of 0.3 mM (*tert*-butyl hydroperoxide for 2 h and 4 h treatments, and 50 benzo(a)pyrene for 24 h treatments. Data are means ± SD from three independent experiments, with 50 cells analysed per experimental point. $^*p < 0.05$, treated *versus* control cells (ANOVA, Kruskal–Wallis with Dunnet's post test).

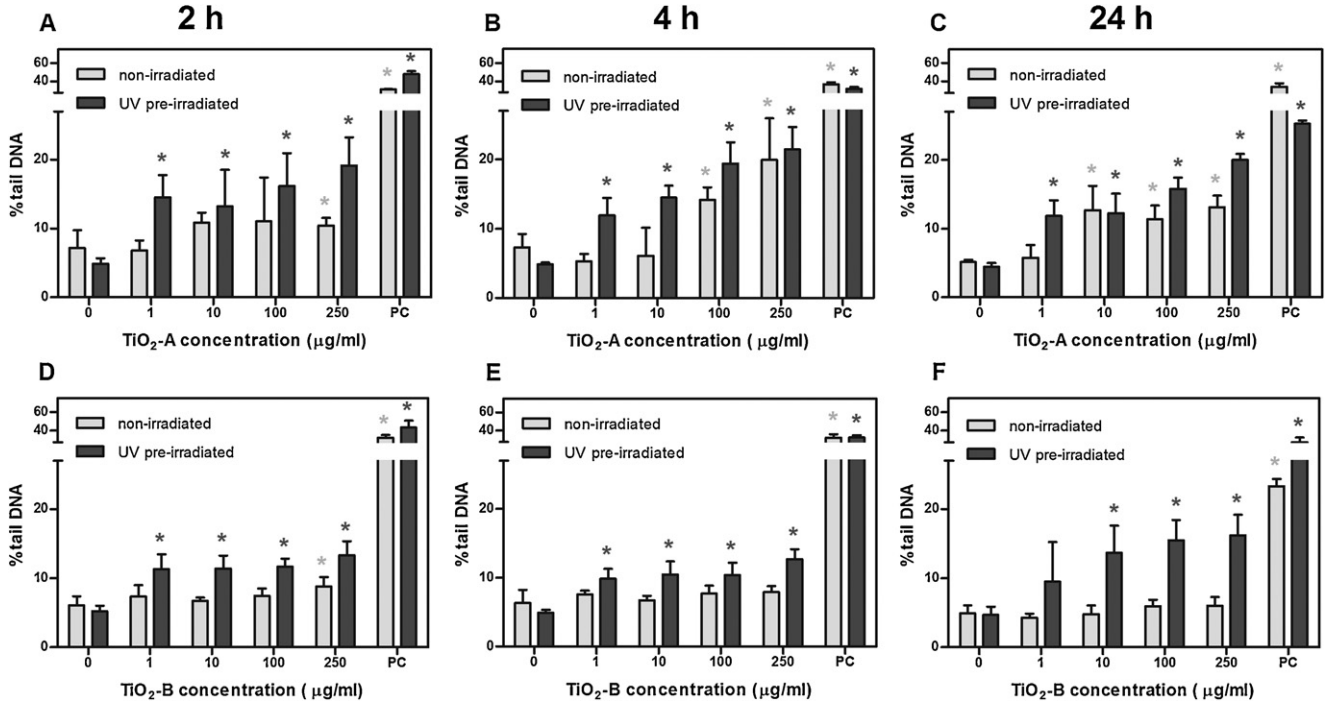

Fig. 6. The effects of non-irradiated and UV pre-irradiated TiO_2-A and TiO_2-B on induction of Fpg-sensitive sites in HepG2 cells, as indicated: after 2 h (A and D), 4 h (B and E) and 24 h (C and F). The modified comet assay was performed as described in Section 2. All further details as for legend to Fig. 5.

In the HepG2 cells exposed to non-irradiated TiO_2-A, there were time-dependent increases in formation of Fpg-sensitive sites, which reached significance ($p < 0.05$) at 2 h exposure with 250 μg/ml (Fig. 6A), at 4 h exposure with 100 μg/ml and 250 μg/ml (Fig. 6B), and at 24 h exposure with concentrations above 10 μg/ml (Fig. 6C). In the cells exposed to UV pre-irradiated TiO_2-A there were significant ($p < 0.05$) dose-dependent increases in Fpg-sensitive sites at all times of exposure (2 h, 4 h and 24 h) and at all applied concentrations (Fig. 6A–C). From these data, it can also be seen that UV pre-irradiated TiO_2-A induced higher levels of Fpg-sensitive sites than non-irradiated TiO_2-A.

Exposure of the HepG2 cells for up to 24 h to non-irradiated TiO_2-B induced significant ($p < 0.05$) increases in Fpg-sensitive sites only after 2 h of exposure at 250 μg/ml (Fig. 6D–F). On the other hand, exposure of the HepG2 cells to UV pre-irradiated TiO_2-B induced significant ($p < 0.05$) increases in Fpg-sensitive sites after 2 h and 4 h exposure at all of the concentrations tested (Fig. 6D and E), while after 24 h exposure there were significant increases at concentrations above 10 μg/ml (Fig. 6F). From these data we can see that UV pre-irradiated TiO_2-B induced much higher levels of Fpg-sensitive sites than non-irradiated TiO_2-B.

The levels of oxidative DNA damage induced after 2 h and 4 h exposure by UV pre-irradiated TiO_2-B (Fig. 6D and E) was lower than that induced by UV pre-irradiated TiO_2-A (Fig. 6A and B), while after 24 h exposure, the levels of oxidative DNA damage were comparable (Fig. 6C and F).

4. Discussion

Due to the numerous applications of TiO_2 photocatalysis, human and environmental exposure to photo-activated TiO_2 particles is very likely. However, to our knowledge, to date, no studies have addressed the possibility that photo-activated TiO_2 might also have greater reactivity, and consequently greater toxicity, after the UV irradiation has been discontinued. In the present study, we compared the toxic effects of non-irradiated and UV pre-irradiated anatase TiO_2 particles of different average particle sizes, TiO_2-A (18 nm) and TiO_2-B (105 nm), in the experimental model of HepG2 cells. We showed that the toxic potential of these TiO_2 particles after UV pre-irradiation is drastically increased not only for the TiO_2-A NPs, but also for submicron-sized TiO_2-B particles. Non-irradiated TiO_2 particles did not affect survival of the cells, even at relatively high concentrations and after long times of exposure (48 h), which is in agreement with a number of other studies, for TiO_2 NPs [9,16,17,32–35], as well as for submicron-sized TiO_2 particles [4–6,35]. Conversely, UV pre-irradiated TiO_2 particles of both sizes significantly decreased HepG2 cell viability at all times of exposure, and after UV pre-irradiation their genotoxic potential also increased.

Many studies in the literature have suggested that TiO_2 particles larger than 100 nm are biologically inert [5,6,36], while TiO_2 NPs show greater cytotoxic and genotoxic potential. There is a general hypothesis that the smaller the particle is, the higher the potency it has to induce toxicity in the absence of photo-activation, which has been confirmed in various cell types [4,32,37–39]. However, in the present study, the coarser particles, TiO_2-B, non-irradiated and UV pre-irradiated, induced similarly high levels of DNA strand breaks in HepG2 cells as the TiO_2-A NPs. Although the primary crystallites in TiO_2-B particles are larger than in TiO_2-A, the genotoxic effects of the TiO_2-B particles can be explained by their colloidal behaviour. We showed that the two TiO_2 particles used in this study behave significantly differently in water, while the differences observed in cell-growth medium are relatively small. It is likely that not only crystallite size, but especially the form of the particles (strongly aggregated or softly granulated) and its colloidal properties (zeta potential, dispersibility) will influence their biological effects. The zeta potential and also the dispersibility of the TiO_2-B particles are much higher and might increase its bioavailability, and hence its induction of DNA damage. On the other hand, oxidative DNA damage was induced by non-irradiated TiO_2-A, but not TiO_2-B, which is most probably a consequence of the observed

difference in size of the crystallites (18 nm for TiO_2-A, and 105 nm for TiO_2-B) and the specific surface areas (129.3 m^2/g for TiO_2-A, and 8.6 m^2/g for TiO_2-B). Although TiO_2-A particles are agglomerated and aggregated, some of them are still free, and because of their small size and larger specific surface area, they are more reactive and cause more damage than larger TiO_2-B particles. Gurr et al. [4], who studied the same particles as in the present study in terms of TiO_2-B (Sigma T8141), showed that without UV irradiation, these TiO_2 particles did not induce oxidative stress, while anatase TiO_2 NPs (10 and 20 nm) did. They also measured the particle sizes in cell-growth medium and found that TiO_2 NPs produced aggregations of 1000 nm in diameter, while the >100 nm particles showed no aggregation, which is in agreement with our observations here. Another possible explanation is the different chemical compositions of the surface layers of the particles that were indicated by the difference seen in the natural pH of the two particles. In addition, a decrease in the zeta potential and an increase in conductivity over time indicate a degree of solubility of the particles.

However, after UV irradiation, TiO_2-A and TiO_2-B resulted in higher and comparable oxidative DNA damage, because the photocatalytic effects predominate now, such that their toxic potential is not significantly different any more. As already mentioned, a number of studies have shown that in the presence of UV irradiation TiO_2 is more cytotoxic and genotoxic than in the absence [18–21,40–43]. Nakagawa et al. [16] studied genotoxicity of anatase and rutile nano-sized and submicron-sized TiO_2 in the presence and absence of UV irradiation using several in vitro genotoxicity assays (a comet assay and cell mutation assay with mouse lymphoma cells, a microbial mutation assay with *Salmonella typhimurium*, and a chromosomal aberration assay with Chinese hamster cells). Without UV irradiation, the nano-sized and submicron-sized TiO_2 particles showed little or no genotoxicity, while in combination with UV irradiation, the TiO_2 particles showed significant genotoxicity, with nano-sized anatase TiO_2 as the most potent. Similar results were obtained in a study by Reeves et al. [17], who in a test system with goldfish skin cells showed that anatase TiO_2 NPs alone do not affect cell viability, although they do cause oxidative DNA damage. The combination of TiO_2 NPs with UV showed a significant dose-dependent decrease in cell viability and further increases in oxidative DNA damage. They also showed that the toxic/genotoxic effects they observed were most likely due to the formation of hydroxyl radicals. Similar findings were reported by Uchino et al. [19] in a test system with Chinese hamster ovary cells. They also observed that UV irradiation of anatase TiO_2 NPs produced larger numbers of hydroxyl radicals in comparison to rutile TiO_2 NPs. The conclusion from all of these studies was that photo-activated TiO_2 is more toxic than non-photo-activated TiO_2. However, in these studies in which simultaneous exposure of cells to UV and TiO_2 was used, it is not possible to exclude any contributions of the UV irradiation *per se* to the increased toxic effects observed.

Our study has several implications that need to be considered in the future evaluation of the potential toxicity of TiO_2 particles. The first and most important is related to our finding that irrespective of the particle size, photo-activated anatase TiO_2 particles retained elevated reactivity even after the termination of UV exposure. This was seen as greater cytotoxicity and genotoxicity, and in particular, oxidative DNA damage. Among the mechanisms responsible for observed toxic effects, the generation of ROS after UV irradiation is definitely the greatest. Exposure to UV has enough energy to excite electrons from the valence band to conduction band resulting in formation highly reactive electron–hole pairs [13]. In aqueous environments electron (e^-) reduces oxygen to give superoxide anion radicals, which can be dismutated to hydrogen peroxide, and the hole oxidizes water to give hydroxyl radical, respectively. Apparently the UV-irradiated TiO_2 particles remained in excited stage also after the irradiation was discontinued, which can explain their higher toxic and genotoxic potential compared to non-irradiated TiO_2. From what we have also seen from our data, the toxicity and genotoxicity of photo-activated TiO_2 is not dependent on particle size (at least in the investigated range of particle sizes), since both sizes of TiO_2 used in our study (TiO_2-A < 100 nm, and TiO_2-B >100 nm) showed comparable cytotoxic and genotoxic potential. Also, coarse-sized anatase TiO_2 without or with photo-activation cannot be generally considered as safer than nano-sized anatase TiO_2. Our data show that quite different particles can behave relatively similarly in the cell-growth medium, and that not only crystallite size, but also the form of the particles (strongly aggregated or softly granulated) and their colloidal properties (zeta potential, dispersibility) influence their biological effects. This gives us a whole new perspective on the behaviour of TiO_2 particles of different sizes in different media, and this will have to be taken into account in any further safety evaluations.

5. Conclusions

The results of the present study can be considered relatively alarming and they promote concerns for the safety of all biological systems that might be exposed to photo-activated TiO_2 almost anywhere. Considering that 90–99% of the UV light that reaches the surface of the earth shows a UV spectrum with wavelengths of 320–400 nm, almost all of the TiO_2 present in the environment that is not specifically protected from this UV can be photo-activated, and become potentially more dangerous in comparison to non-photo-activated TiO_2. Even more worrying, UVA penetrates deep into the skin [44], and as such the users of sunscreens that contain TiO_2 can actually be exposed to the more dangerous, photo-activated TiO_2. To our knowledge, this aspect has not been addressed appropriately, as in studies carried out to date only short-term concurrent exposure to UV and TiO_2 has been evaluated. In addition, the possibilities of the application of TiO_2 photocatalytic properties are enormous, which will contribute further to increased environmental and human exposure to photo-activated TiO_2. Since the literature contains no similar studies, it is very important to evaluate the toxic potential of photo-activated TiO_2 in different experimental models.

Our data also change the generally accepted concepts about the toxicity of TiO_2 NPs and about the 'inertness' of larger TiO_2 particles. We have shown that cytotoxity and genotoxicity of TiO_2 anatase particles after UV irradiation drastically increases irrespective of particle size. Our new approach that avoids direct UV irradiation of the cells is an appropriate model for toxicological studies of photo-activated TiO_2, and also of other materials that can be photo-activated, and that might provide more reliable evaluation of their potential toxicity. We thus recommend the described experimental approach for inclusion in future studies of the toxicological properties of photo-activated materials.

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References

[1] H. Nordman, M. Berlin, Titanium, in: L. Friberg, G.F. Nordberg, V.B. Vouk (Eds.), Handbook on the Toxicology of Metals, Elsevier, Amsterdam, 1986, pp. 595–609.
[2] M.C. Lomer, R.P. Thompson, J.J. Powell, Fine and ultrafine particles of the diet: influence on the mucosal immune response and association with Crohn's disease, Proc. Nutr. Soc. 61 (2002) 123–130.

[3] C. Gelis, S. Girard, A. Mavon, M. Delverdier, N. Paillous, P. Vicendo, Assessment of the skin photoprotective capacities of an organo-mineral broad-spectrum sunblock on two *ex vivo* skin models, Photodermatol. Photoimmunol. Photomed. 19 (2003) 242–253.
[4] J.R. Gurr, A.S. Wang, C.H. Chen, K.Y. Jan, Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells, Toxicology 213 (2005) 66–73.
[5] J.L. Chen, W.E. Fayerweather, Epidemiologic study of workers exposed to titanium dioxide, J. Occup. Med. 30 (1988) 937–942.
[6] B.K. Bernard, M.R. Osheroff, A. Hofmann, J.H. Mennear, Toxicology and carcinogenesis studies of dietary titanium dioxide-coated mica in male and female Fischer 344 rats, J. Toxicol. Environ. Health 29 (1990) 417–429.
[7] Q. Rahman, M. Lohani, E. Dopp, H. Pemsel, L. Jonas, D.G. Weiss, et al., Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts, Environ. Health Perspect. 110 (2002) 797–800.
[8] Y. Cui, X. Gong, Y. Duan, N. Li, R. Hu, H. Liu, et al., Hepatocyte apoptosis and its molecular mechanisms in mice caused by titanium dioxide nanoparticles, J. Hazard. Mater. 183 (2010) 874–880.
[9] S. Hackenberg, G. Friehs, K. Froelich, C. Ginzkey, C. Koehler, A. Scherzed, et al., Intracellular distribution, geno- and cytotoxic effects of nanosized titanium dioxide particles in the anatase crystal phase on human nasal mucosa cells, Toxicol. Lett. 195 (2010) 9–14.
[10] J. Petković, B. Žegura, M. Stevanović, N. Drnovšek, D. Uskoković, S. Novak, M. Filipič, DNA damage and alterations in expression of DNA-damage-responsive genes induced by TiO_2 nanoparticles in human hepatoma HepG2 cells, Nanotoxicology 5 (2011) 341–353.
[11] J.J. Wang, B.J. Sanderson, H. Wang, Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells, Mutat. Res. 628 (2007) 99–106.
[12] R. Konaka, E. Kasahara, W.C. Dunlap, Y. Yamamoto, K.C. Chien, M. Inoue, Irradiation of titanium dioxide generates both singlet oxygen and superoxide anion, Free Radic. Biol. Med. 27 (1999) 294–300.
[13] A. Fujishima, T.N. Rao, D.A. Tryk, Titanium dioxide photocatalysis, J. Photochem. Photobiol. C: Photochem. Rev. 1 (2000) 1–21.
[14] K. Hashimoto, H. Irie, A. Fujishima, TiO_2 photocatalysis: a historical overview and future prospects, Jpn. J. Appl. Phys. 44 (2005) 16.
[15] Z. Zhang, C.-C. Wang, R. Zakaria, J.Y. Ying, Role of particle size in nanocrystalline TiO_2-based photocatalysts, J. Phys. Chem. B 102 (1998) 10871–10878.
[16] Y. Nakagawa, S. Wakuri, K. Sakamoto, N. Tanaka, The photogenotoxicity of titanium dioxide particles, Mutat. Res. 394 (1997) 125–132.
[17] J.F. Reeves, S.J. Davies, N.J.F. Dodd, A.N. Jha, Hydroxyl radicals (OH) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells, Mutat. Res. Fundam. Mol. Mech. Mutagen. 640 (2008) 113–122.
[18] R.C. Gopalan, I.F. Osman, A. Amani, M. De Matas, D. Anderson, The effect of zinc oxide and titanium dioxide nanoparticles in the Comet assay with UVA photoactivation of human sperm and lymphocytes, Nanotoxicology 3 (2009) 33–39.
[19] T. Uchino, H. Tokunaga, M. Ando, H. Utsumi, Quantitative determination of OH radical generation and its cytotoxicity induced by TiO_2-UVA treatment, Toxicol. In Vitro 16 (2002) 629–635.
[20] M. Cho, H. Chung, W. Choi, J. Yoon, Linear correlation between inactivation of *E. coli* and OH radical concentration in TiO_2 photocatalytic disinfection, Water Res. 38 (2004) 1069–1077.
[21] C. Wang, S. Cao, X. Tie, B. Qiu, A. Wu, Z. Zheng, Induction of cytotoxicity by photoexcitation of TiO_2 can prolong survival in glioma-bearing mice, Mol. Biol. Rep. 38 (2011) 523–530.
[22] H. Ikehata, K. Kawai, J.-I. Komura, K. Sakatsume, L. Wang, M. Imai, et al., UVA1 genotoxicity is mediated not by oxidative damage but by cyclobutane pyrimidine dimers in normal mouse skin, J. Invest. Dermatol. 128 (2008) 2289–2296.
[23] C. Kielbassa, B. Epe, DNA damage induced by ultraviolet and visible light and its wavelength dependence, Method Enzymol. 319 (2000) 436–445.
[24] G.M. Halliday, Inflammation, gene mutation and photoimmunosuppression in response to UVR-induced oxidative damage contributes to photocarcinogenesis, Mutat. Res./Fundam. Mol. Mech. Mutagen. 571 (2005) 107–120.
[25] A.R. Collins, M. Dusinska, C.M. Gedik, R. Stetina, Oxidative damage to DNA: do we have a reliable biomarker? Environ. Health Perspect. 104 (1996) 465–469.
[26] B. Tryba, Increase of the photocatalytic activity of TiO_2 by carbon and iron modifications, Int. J. Photoenergy 8 (2008) 15.
[27] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[28] B. Zegura, B. Sedmak, M. Filipic, Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2, Toxicon 41 (2003) 41–48.
[29] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.
[30] B. Žegura, M. Filipič, Application of an *in-vitro* comet assay for genotoxicity testing, in: Y. Zhengyin, G.W. Caldwell (Eds.), Methods in Pharmacology and Toxicology, Optimization in Drug Discovery: In Vitro Methods, Humana Press, Totowa, 2004, pp. 301–313.
[31] D.L. Liao, G.S. Wu, B.Q. Liao, Zeta potential of shape-controlled TiO_2 nanoparticles with surfactants, Colloid. Surf. A: Physicochem. Eng. Aspects 348 (2009) 270–275.
[32] S.M. Hussain, K.L. Hess, J.M. Gearhart, K.T. Geiss, J.J. Schlager, *In-vitro* toxicity of nanoparticles in BRL 3A rat liver cells, Toxicol. In Vitro 19 (2005) 975–983.
[33] S. Kim, J.E. Choi, J. Choi, K.-H. Chung, K. Park, J. Yi, et al., Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells, Toxicol. In Vitro 23 (2009) 1076–1084.
[34] H.A. Jeng, J. Swanson, Toxicity of Metal Oxide Nanoparticles in Mammalian Cells, J. Environ. Sci. Health A: Toxic Hazard. Subst. Environ. Eng. 41 (2006) 2699–2711.
[35] S. Wagner, S. Münzer, P. Behrens, T. Scheper, D. Bahnemann, C. Kasper, Cytotoxicity of titanium and silicon dioxide nanoparticles, J. Phys. Conf. Ser. 170 (2009) 012022.
[36] G.A. Hart, T.W. Hesterberg, *In-vitro* toxicity of respirable-size particles of diatomaceous earth and crystalline silica compared with asbestos and titanium dioxide, J. Occup. Environ. Med. 40 (1998) 29–42.
[37] T.C. Long, N. Saleh, R.D. Tilton, G.V. Lowry, B. Veronesi, Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity, Environ. Sci. Technol. 40 (2006) 4346–4352.
[38] T. Xia, M. Kovochich, J. Brant, M. Hotze, J. Sempf, T. Oberley, et al., Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm, Nano Lett. 6 (2006) 1794–1807.
[39] T. Uchino, Y. Ikarashi, T. Nishimura, Effects of coating materials and size of titanium dioxide particles on their cytotoxicity and penetration into the cellular membrane, J. Toxicol. Sci. 36 (2011) 95–100.
[40] R. Cai, Y. Kubota, T. Shuin, H. Sakai, K. Hashimoto, A. Fujishima, Induction of cytotoxicity by photoexcited TiO_2 particles, Cancer Res. 52 (1992) 2346–2348.
[41] Y. Kubota, T. Shuin, C. Kawasaki, M. Hosaka, H. Kitamura, R. Cai, et al., Photokilling of T-24 human bladder cancer cells with titanium dioxide, Br. J. Cancer. 70 (1994) 1107–1111.
[42] P.C. Maness, S. Smolinski, D.M. Blake, Z. Huang, E.J. Wolfrum, W.A. Jacoby, Bactericidal activity of photocatalytic TiO_2 reaction: toward an understanding of its killing mechanism, Appl. Environ. Microbiol. 65 (1999) 4094–4098.
[43] W.G. Wamer, J.J. Yin, R.R. Wei, Oxidative damage to nucleic acids photosensitized by titanium dioxide, Free Radic. Biol. Med. 23 (1997) 851–858.
[44] R. Raghavan, J. Joseph, Ultraviolet and visible spectrophotometry in pharmaceutical analysis, in: J. Swarbrick, J.C. Boylan (Eds.), Encyclopedia of Pharmaceutical Technology, Marcel Dekker, New York, 1997, pp. 292–295.