Shape-engineered titanium dioxide nanoparticles (TiO2-NPs): cytotoxicity and genotoxicity in bronchial epithelial cells

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In conclusion, cytotoxicity of NPs was low, influenced by shape as well as by light exposure. Instead, genotoxicity seemed to be influenced by cellular-uptake and aggregation tendency. This study suggest that shape engineered TiO2-NPs are safer than the commercial ones.
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Highlights

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Highlights

- Cytotoxicity/genotoxicity evaluation of engineered TiO$_2$-NPs with different shapes
- TiO$_2$-NPs cytotoxicity was low, influenced by the shape and by light exposure
- Genotoxicity was influenced by cellular-uptake and aggregation tendency of TiO$_2$-NPs
- The presence of light enhanced the oxidative DNA damage
- It seems that shape engineered TiO$_2$-NPs are safer than the commercial ones
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List of Abbreviations
BEAS-2B – Human bronchial epithelial cells
$D_h$ – Hydrodynamic diameter

Fpg – formamidopyrimidine glycosylase

NP – Nanoparticle

$\text{TiO}_2$ – Titanium dioxide
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The aim of this study was to evaluate cytotoxicity (WST-1 and LDH assays) and genotoxicity (Comet assay) of three engineered TiO$_2$-NPs with different shapes (bipyramids, rods, platelets) in comparison with two commercial TiO$_2$-NPs (P25, food grade).

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Keywords: shape-engineered TiO$_2$ nanoparticles; genotoxic and oxidative damage; Comet assay; cytotoxicity; Raman spectroscopy.
1. Introduction

Nanoparticles (NPs) are defined as particles having their three dimension in the range of 1 – 100 nm (ISO/TS 27687:2008). Actually, many consumer products incorporates NPs. The technological, medical and economic benefits of NPs are considerable, but the presence of nanoparticles in the environment could cause adverse effects to humans. NPs have a grater surface area per unit mass, so they potentially have an increased biological activity compared to fine particles. Moreover, NPs size is comparable to size of cellular structures, so NPs might potentially emulate biological molecules or interfere physically with biological processes (Magdolenova et al. 2012a).

TiO$_2$ is the oxide of titanium and it has different crystalline structures: anatase, brookite and rutile. Brookite is not produced by industry and is not incorporated in commercial products. In contrast, rutile and anatase are largely used in commercial products (Jovanovic 2015). TiO$_2$ is one of the most frequently applied NPs and it is in the top five NPs used in consumer products (Shi et al. 2013). TiO$_2$-NPs produced are used primarily as a pigment owing to their brightness, resistance to discoloration and high refractive index. As a pigment TiO$_2$-NPs are incorporated in paints, plastic materials, paper, foods, medical products and cosmetics. Due to its catalytic and photocatalytic properties, TiO$_2$ is also used as an antimicrobial agent and a catalyst for purification of air and water (Bonetta et al. 2013, Tomankova et al. 2015).

TiO$_2$-NPs could be engineered in terms of shapes and sizes by changing synthesis conditions such as raw material, temperature, acidic and alkaline conditions. Engineered TiO$_2$-NPs with various shapes (e.g. rods, dots and belts) have been prepared for different applications (Bernard and Curtiss 2005, Sha et al. 2015, Wang et al. 2004). In particular engineered fiber-shaped nanomaterials (i.e. nanowires, nanotubes) are very attractive because showed higher activity and advantages in photocatalysis, charge transfer and sensing applications due to
their structure (Hamilton et al. 2009). However, these new and enhanced properties may also induce higher toxicological effects upon exposure with biological tissues.

Humans can be exposed to TiO$_2$-NPs via three portals of entry: oral (mainly via food consumption), dermal (often through cosmetic and sunscreen applications) and inhalation (mainly under occupational and manufacturing conditions) (Warheit and Donner 2015).

Based on the evidence that TiO$_2$ can induce lung cancer in rats, TiO$_2$-NPs were classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC; Kuempel and Ruder 2012). Indeed, the inhalation and instillation of rutile and anatase TiO$_2$-NPs induced lung tumors (Xu et al. 2010), broncho-alveolar adenomas and cystic keratinizing squamous cell carcinomas (De Matteis et al. 2016; Mohra et al. 2006). TiO$_2$-NPs were also classified as potential occupational carcinogens by the National Institute for Occupational Safety and Health (NIOSH 2011; Chen et al. 2014).

Many in vitro studies showed cytotoxicity, genotoxicity and oxidative effects induced by TiO$_2$-NPs through oxidants generation, inflammation and apoptosis (Jugan et al. 2011, Karlsson et al. 2015, Park et al. 2008, Shi et al. 2010). The potential of NPs to cause DNA damage is an important aspect that needs attention because it could induce mutations and carcinogenesis. Physico-chemical characteristics of NPs have an important role on toxicity. Different studies showed that biological effects can be influenced by crystalline structure, size, shape, exterior area, agglomeration/aggregation and surface properties (Bhattacharya et al. 2009, Johnston et al. 2009). Some studies reveled that crystalline structure probably influences the induced toxicity, in particular the anatase seems to be more reactive (Sayes et al. 2006) and induces more toxic, genotoxic and inflammatory effects, than the rutile (Falck et al. 2009, Petkovic et al. 2011, Xue et al. 2010). However, other studies gave contradictory results with rutile forms being more toxic than anatase (Gurr et al. 2005, Numano et al. 2014, Uboldi et al. 2016). The effect of agglomeration/aggregation of NPs on toxicity is not well
understood yet. In recent studies, some authors demonstrated that agglomeration can influence NPs genotoxicity (Magdolenova et al. 2012b, Prasad et al. 2013).

Although physico-chemical properties of NPs can have an important role in the impact on their toxicity, only few studies on shape dependent TiO\textsubscript{2} toxicity has been conducted (Allegri et al. 2016, Hamilton et al. 2009, Park et al. 2013). Additional studies are needed to evaluate the role of shape on TiO\textsubscript{2}-NPs toxicity in order to produce useful data for assessing the safety of engineered NPs. To address this issue, the aim of this study was to investigate cytotoxicity (WST-1 and LDH) and genotoxicity (Comet assay) of three types of engineered TiO\textsubscript{2}-NPs of different shapes (bipyramids, rods and platelet NPs) in BEAS-2B (cells isolated from human bronchial epithelium) in comparison with two commercial types of TiO\textsubscript{2}-NPs (P25 and food grade). Since the exposure to TiO\textsubscript{2}-NPs mainly occurs through respiratory tract, human cells of the respiratory system (such as BEAS-2B), were selected as a good cell model for \textit{in vitro} toxicology tests. All the TiO\textsubscript{2}-NPs in this study were first physico-chemically characterized, even in different culture media to study their agglomeration state, and then they were biologically evaluated. In order to take into account the photocatalytic properties of the TiO\textsubscript{2}-NPs, we investigated the effects on cytotoxicity and genotoxicity on BEAS-2B under light exposure and in darkness. Moreover, a modern application of Raman spectroscopy, the 3D confocal Raman imaging, was used to study the uptake of the NPs within the BEAS-2B cells, as the Raman spectra provide information about both organic molecules and solid NPs simultaneously (Ahlinder et al. 2013).

\section*{2. Materials and methods}

\subsection*{2.1 Synthesis and Preparation of TiO\textsubscript{2} NPs dispersion}

Rods and bipyramids TiO\textsubscript{2}-NPs were synthesized by the forced hydrolysis of an aqueous solution of TiIV(triethanolamine)\textsubscript{2}titanatranes (Ti(TEOA)\textsubscript{2}), using triethanolamine (TEOA)
as shape controller; pH of synthesis was adjusted by adding 1 M NaOH solution; details of these procedures were previously reported (Iannarelli et al. 2016, Lavric et al. 2017). The synthesis of platelet NPs was performed with a solvothermal method (Han et al. 2009, Zhang et al. 2012). In a typical synthesis: a precise volume of Ti(OBu)$_4$ was added in a 150 ml Teflon pot and the desired volume of concentrated hydrofluoric acid was added dropwise under stirring. The Teflon pot was sealed and kept under stirring at high temperature (250°C) for 24h in autoclave. The resulting paste was centrifuged three times and washed with acetone and with water (Milli-Q) to remove the residual organics. The synthesis dispersions were subjected to dialysis process (against ultrapure water, using Spectra/Por dialysis membrane tubing MWCO 8–14 kDa) in order to clean the medium. To avoid agglomeration and precipitation, dimethylsulfoxide (DMSO 1% in water) was added to the NPs dispersions and the dispersions were homogenized using an ultra-sonication procedure, as previously described by Iannarelli et al. (2016).

The same ultra-sonication procedure was employed in the preparation of the dispersion of commercial TiO$_2$ powders, which were the P25 NPs (Evonik), extensively used in toxicity studies (Karlsson et al. 2015, Magdolenova et al. 2014, Valant et al. 2012), and the food grade NPs (Faravelli Group), incorporated in many edible products (Weir et al. 2012).

Powders of commercial TiO$_2$-NPs were first dispersed in a solution of DMSO (1% in water) and then ultrasonicated as described above.

Stock solutions of both commercial and engineered TiO$_2$-NPs were prepared at the final concentration of 2.5 mg/ml.

2.2 Scanning Electron Microscopy (SEM) including Transmission Mode (T-SEM)

The dimensional characterization (size and shape) of TiO$_2$-NPs was carried out with SEM using a Zeiss Supra 40 instrument (Zeiss) equipped with a Schottky field emitter, the standard secondary electrons, i.e. Everhart-Thornley, detector and a high-resolution In-lens detector.
The surface-sensitive In-lens SEM mode better suited to morphological/shape analysis and transmission mode in SEM (T-SEM) better suited for dimensional measurements were applied complementary to the same field of view on the sample.

2.3 Dynamic Light Scattering (DLS) analysis

Delsa Nano™ C Analyzer (Beckman Coulter) equipped with a 638 nm diode laser and a temperature control was used for the DLS measurements. The laser fluctuation was detected on a photomultiplier tube detector positioned behind the cuvette with an angle of 163°. Hydrodynamic diameters were calculated setting temperature at 25°C, viscosity (η) 0.890 cP and refractive index of water 1.3325. In order to simulate the culture medium conditions, DLS analyses were conducted on dilution of TiO₂ dispersions (1:4) in a 1% DMSO aqueous solution, as reference analysis, and in base and complete RPMI 1640 medium.

2.4 Raman spectroscopy analysis

The aqueous suspensions of the TiO₂-NPs under investigation were freeze-dried to obtain a solid powder. Raman spectroscopy was used in the analysis of dry TiO₂-NPs powder using a DXR™ Raman Microscope (Thermo Scientific) with a laser wavelength at 532 nm, a laser power of 1 mW and a 10x microscope objective. Spectra were collected in the 50–1800 cm⁻¹ spectral region, with a grating resolution of 3.3–3.9 cm⁻¹, exposure time of 1 s and 20 scans in total.

2.5 Cell culture and exposure

BEAS-2B cells, isolated from human bronchial epithelium, were obtained from the ATCC (American Type Culture Collection). BEAS-2B were grown, maintained and treated in completed RPMI 1640 medium (37°C, 5% CO₂).

Just before the exposure the fresh stock solution of NPs (2.5 mg/ml) was vortexed and sonicated (30 min) in order to disperse the NPs. NPs (5 – 160 µg/ml) were added to the cells and mixed on a shaker (10 min). The cells were exposed for 1h under laboratory light
(36W/840 Lumilux Cool White-36 W, 3350 lm, 4000 K-supplied from OSRAM lighting AG) and then incubated at 37 °C in darkness (23h) (exposure with light). To quantify effects due to the photocatalytic activity of TiO$_2$, cells were exposed for 24h in darkness (exposure in darkness).

After exposure, cytotoxicity and genotoxicity assays were performed.

2.6 Cytotoxicity

Cell viability was assessed using Cell Proliferation Reagent WST-1 (Roche). The assay was performed as previously described by Gea et al. (2018). Briefly, BEAS-2B were seeded in 24-well plates (5×10$^4$ cells/well) and exposed to NPs (5, 10, 20, 50 and 80 µg/ml, equivalent to 1.3, 2.6, 5.2, 13.0, 20.7 µg/cm$^2$). After exposure, WST-1 was added (50 µl/well) and incubated for 3h (37 °C). After incubation, well contents were centrifuged and the supernatants were transferred in 96-well plate to remove the interference owing to the NPs. The absorbance was measured at 440 nm (Tecan Infinite Reader M200 Pro). Absorbance of unexposed cells was used as negative control. Data were expressed as a percentage of viability. All experiments were performed in quadruplicate (four wells for each experimental condition).

As indicator of cell membrane damage, Lactate dehydrogenase activity was measured in cell-free culture supernatants using the LDH assay kit (Cytotoxicity Detection Kit PLUS, Roche) modified for NPs exposure. Briefly, BEAS-2B cells were seeded in 24-well plates (5 × 10$^4$ cells/well) and exposed to NPs (5, 10, 20, 50 and 80 µg/ml, equivalent to 1.3, 2.6, 5.2, 13.0, 20.7 µg/cm$^2$). After exposure, the contents of each well were centrifuged to remove the interference owing to the NPs. Each supernatant (100 µl) was transferred into 96-well plate, mixed with Reaction Mixture (100 µl/well) and incubated for 30 min at 15 – 25 °C. After incubation, Stop Solution (50 µl/well) was added and the absorbance was measured at 490 nm (Tecan Infinite Reader M200 Pro). Absorbance measurement of unexposed cells was
used as negative control, while absorbance measurement of unexposed lysed cells was used as positive control. LDH release was expressed as a percentage of control cells. All experiments were performed in triplicate (three wells for each experimental condition).

2.7 Genotoxicity

For DNA damage evaluation the BEAS-2B cells were cultured overnight in 6-well plates (3 × 10^5 cells/well) before exposure to NPs. Cells were exposed to different doses of NPs: 20, 50, 80, 120 and 160 µg/ml, equivalent to 5.2, 13.0, 20.8, 31.2, 41.6 µg/cm². Unexposed cells and cells treated with NPs dispersion liquid (DMSO 1%) were used as negative controls. After exposure, cell viability was determined using trypan blue staining. The Comet assay was performed according to Tice et al. (2000) after slight modifications (Bonetta et al. 2009). The percentage of tail intensity was used to estimate DNA damage. A hundred randomly selected cells per treatment (2 spot) were analyzed using the Comet Assay IV software (Perceptive Instruments Ltd). Two independent experiments were performed for each experimental condition.

The oxidative DNA damage was evaluated using the formamidopyrimidine glycosylase (Fpg)-modified Comet assay as reported in Bonetta et al. (2018). For each experimental point, the mean % tail intensity from enzyme untreated cells (direct DNA damage) and mean % tail intensity for Fpg-enzyme treated cells (direct and indirect DNA damage) were calculated. Two independent experiments were performed for each experimental condition.

2.8 3D confocal micro-Raman imaging spectroscopy

Raman grade Calcium fluoride (CaF₂) windows (Crystran Tachnology srl) were employed as alternative substrate instead of standard plastic substrates for cells growing due to the low toxicity and almost absent background signals (Kann et al. 2015). The BEAS-2B cells were cultured overnight in 6-well plates on a CaF₂ substrate (3 × 10^5 cells/well) before exposure to
NPs. Cells were treated with NPs (80 µg/ml, 24h). After exposure, cells were washed twice with PBS and fixed with 3 ml of methanol. CaF$_2$ substrates were dried and stained with Giemsa dye (4% Giemsa’s azur eosin methylene blue solution, 4% Sorensen buffer 0.067 M pH 6.8, 8 min at room temperature), then washed twice with distilled water and dried. Giemsa staining is one of the standard procedures in histology, useful to evidence morphological cells features, such as cell nuclei, which appear in various shades of red/purple, and the cytoplasm, which appears blue.

3D confocal micro-Raman imaging spectroscopy of BEAS-2B cells was conducted with a DXR™xi Raman Imaging Microscope (Thermo Scientific) using a laser wavelength at 532 nm, a 1 mW laser power, a 100X microscope objective and a motorized stage with a 1 µm of step size and a 1 µm offset. Spectra were collected in the 50–3500 cm$^{-1}$ spectral region with a grating resolution of 5 cm$^{-1}$, an exposure time of 0.025 s and 5 scans in total. 3D Raman images were reconstructed taking the Raman peaks at 1600 cm$^{-1}$ of methylene blue and the $E_g$ band at 144 cm$^{-1}$ of the TiO$_2$-NPs, respectively. Each cell was investigated at different focal planes and a chemical image was obtained by the combination of the $\nu$(C-C)ring at 1600 cm$^{-1}$ of the methylene blue and the $E_g$ band at 144 cm$^{-1}$ of the TiO$_2$-NPs. Since methylene blue is contained in the Giemsa stain and it is widely distributed into the fixed cells, its signals were considered representative of the entire volume of the cells. As far as the tracking of the NPs are concerned, the $E_g$ band at 143 cm$^{-1}$ is the most intense signal in the molecular fingerprint of the anatase TiO$_2$ and the region between 50 cm$^{-1}$ and 400 cm$^{-1}$ in the Raman spectrum is usually free of the vibrational bands of biological species. Therefore, this signal was selected to sensitively locate the TiO$_2$-NPs inside the cells. Image J software was used in the development of the 3D chemical images both for cells and TiO$_2$-NPs, which were superimposed using a Solidworks® 2016 Cad based software. 3D Raman chemical images are presented using a color meshwork i.e. blue for cell tissues and red for TiO$_2$ agglomerates.
2.9 Statistical analysis

IBM SPSS software (ver. 24.0) was used to perform statistical analysis. The results of WST-1, LDH and Comet assays are presented as the mean±standard deviation. Differences between exposed and control cells were tested by ANOVA followed by Dunnett’s test procedure. Differences between light and dark exposure were tested by ANOVA, followed by the Tukey’s test procedure. Data were considered statistically different for a p-value less than 0.05.

3. Results

3.1 Raman characterization of NPs and size distribution

In order to establish a relationship among the physico-chemical features of NPs and their ability to induce a toxic effect, well-defined and controlled protocols were developed for the production of engineered anatase TiO₂-NPs with different shapes. All the NPs produced in this study were first characterized with a SEM equipped with a transmission-unit for T-SEM, which provided information both on the shape and the size of the constituent NPs (Fig. 1a-e). The Fig. 1 and Table 1 show shapes and particle size of commercial TiO₂-NPs and fabricated engineered TiO₂-NPs.

These NPs were also characterized by Dynamic Light Scattering (DLS) as a quick method for sizing and determining the state of NP agglomeration. For each kind of sample, the agglomeration in 1% DMSO aqueous solution, in base RPMI and complete RPMI (Fig. 1f-j) were compared. In all the TiO₂ materials considered for this study, the agglomeration state increase in base RPMI, while the size distribution in DMSO and in complete RPMI is quite similar.
The crystalline composition of the TiO$_2$-NPs, analyzed by Raman spectroscopy, showed a typical fingerprint of the anatase TiO$_2$ (Fig. S.1) with the characteristic phonon bands $E_g$ at 143 cm$^{-1}$, $E_g$ at 197 cm$^{-1}$, $A_{1g}$ at 397 cm$^{-1}$, $B_{1g}$ at 515 cm$^{-1}$ and $E_g$ at 639 cm$^{-1}$ for all the investigated NPs. Since P25 is a known mixture of anatase and rutile (5:1), with also a small amount of amorphous TiO$_2$ (Ohtani et al. 2010), its Raman spectrum still retains all the typical anatase Raman bands but it also contains two small shoulders at 450 cm$^{-1}$ and 600 cm$^{-1}$, which were assigned to the $E_g$ and $A_{1g}$ phonon bands, respectively, of rutile (Tompsett et al. 1995). All the physiochemical properties of the TiO$_2$-NPs under study such as shape, particle size, hydrodynamic diameter in different liquid media and the crystalline phase are summarized in Table 1.

3.2 Cytotoxicity

The results of the effects of different TiO$_2$-NPs concentration on cell viability (WST-1 assay) are reported in Fig. 2a (exposure with light) and in Fig. 2b (exposure in darkness).

In general, a low cytotoxic effect was observed at the tested doses both in the exposure with light and in the exposure in darkness. The observed viability ranged from 102.8 to 88.4% for the exposure with light and from 99.6 to 87.4% for the exposure in darkness.

Considering the exposure with light, the commercial P25 induced a slight decrease in viability starting from the doses of 50 µg/ml (p<0.05) while no cytotoxic effects were observed for the other commercial NPs (food grade) at the tested concentrations. As far as engineered NPs are concerned, bipyramids and platelet NPs induced the same cytotoxic effect of commercial P25 NPs; on the contrary, rods is the NP shape with higher cytotoxic effect showing a viability decrease already starting from 10 µg/ml (p<0.05 or p<0.001).

Considering the exposure in darkness, a lower cytotoxic effect was observed for commercial P25 NPs with respect to light exposure because a slight decrease in viability was observed for P25 NPs only at the highest dose (80 µg/ml) (p<0.05). As reported after exposure with light,
no cytotoxic effect was observed for the other commercial NPs (food grade). About engineered NPs, the exposure in darkness did not modify the cytotoxic effect of bipyramids NPs resulting in a viability reduction starting from the dose of 50 µg/ml (p<0.001) as reported in the experiment with light. In contrast, in the darkness, rods NPs showed a lower cytotoxic effect than observed with light because a slight decrease in viability was observed for rod NPs only starting from the dose of 20 µg/ml (p<0.05 or p<0.001). As during the exposure with light, platelet NPs induced a decrease in viability; the cytotoxic effect was significant starting from a less dose (10 µg/ml, p<0.05) than in the experiment with light (50 µg/ml).

The results of the effects of different TiO$_2$-NPs concentration on lactate dehydrogenase activity (LDH assay) has been reported in Fig. 2c (exposure with light) and in Fig. 2d (exposure in darkness).

No significant signs of cytotoxicity were seen by the LDH assay in both exposure protocols (with light or in darkness), confirming the low cytotoxic effect evidenced by WST-1 assay.

3.3 Genotoxicity

The results of genotoxic effect and oxidative DNA damage induced by different concentration of NPs are reported in Fig. 3.

Considering the exposure with laboratory light, no genotoxic effect was showed in enzyme untreated cells (direct DNA damage) for commercial P25 NPs (Fig. 3a). On the other hand, a dose-dependent increase of DNA damage was observed for these NPs in enzyme treated cells (direct and indirect DNA damage) respect to the control cells (p<0.05 or p<0.001), with the exception of the last dose (160 µg/ml) that induced a DNA damage equal to 80 µg/ml. A significant oxidative damage was observed for P25 NPs starting from 50 µg/ml (p<0.05 or p<0.001). The results obtained with the other commercial NPs (food grade)(Fig. 3b) showed the presence of a significant dose-response DNA damage both in enzyme untreated cells and
in enzyme treated cells starting from 50 µg/ml. Moreover, the difference between the two
effects resulted significant starting from 20 µg/ml (p<0.05 or p<0.001) highlighting an
oxidative damage induced by food grade NPs.

Respect to commercial NPs, engineered NPs showed a lower extent of DNA damage. In
particular, neither genotoxic effect nor oxidative damage were observed for engineered
bipyramids and rods NPs (Fig. 3c,d). Platelet NPs induced a significant DNA damage respect
to the control cells (p<0.05 or p<0.001) both in enzyme untreated cells and in enzyme treated
cells and they induced a significant oxidative DNA damage starting from 80 µg/ml (p<0.001)
(Fig. 3e). However in contrast with commercial NPs (food grade), a dose-response of the
effects were not observed.

As demonstrated by other authors (Kalsson 2010, Karlsson et al. 2015), an interference
during the scoring of the assay was detected in particular at the higher doses of P25 and
platelet NPs, indeed nanoparticles with some autofluorescence were visible in the comets
“head” and the stained DNA appeared faded. The interference probably caused the loss of
concentration-dependent increase in DNA direct and oxidative damage observed for the
higher doses. The phenomenon could be explained also considering that base oxidation is
hard to measure accurately when there are a lot of strand breaks, because the Comet assay
becomes saturated (Collins et al. 2017).

In order to evaluate the role of the light on the genotoxic and oxidative damage induced by
commercial and engineered NPs, the highest doses (80, 120, 160 µg/ml) of NPs that showed
a genotoxic effect (P25, food grade and platelet NPs) were tested in darkness (24h).

Considering the exposure in darkness, no genotoxic effect was observed for commercial P25
NPs in enzyme untreated cells (direct DNA damage) (Fig. 3f) as reported in the experiment
with light (Fig. 3a). However, in the enzyme treated cells a dose-response DNA damage
(direct and indirect DNA damage) was observed with respect to control cells (p<0.05 or
p<0.001), but oxidative DNA damage was lower than in the experiment with light (p<0.05 or p<0.001). The commercial food grade NPs induced a significant dose-response DNA damage both in enzyme untreated cells and in enzyme treated cells (p<0.001 and p<0.05 respectively) (Fig. 3g). However, the DNA damage resulted in both cases lower than in the experiment with light p<0.05 or p<0.001) and an oxidative damage was induced only at the highest dose (160 µg/ml) (p<0.05).

With regard to engineered NPs, platelet NPs induced a significant DNA damage with respect to the control cells (p<0.05 or p<0.001) both in enzyme untreated cells and in enzyme treated cells (Fig. 3h). However, while the DNA damage in enzyme untreated cells was equivalent to the DNA damage induced in the experiment with light (Fig. 3e), a decrease of DNA damage in enzyme treated cells was observed, resulting in no oxidative damage induced by platelet NPs in darkness (Fig. 3h).

3.4 Confocal micro-Raman spectroscopy

Confocal micro-Raman imaging spectroscopy was used to evaluate the uptake and the distribution of the different types of TiO$_2$-NPs within the cells. 3D chemical images are built by superimposing the different maps of each cell at their corresponding focal planes and they are presented using a color meshwork i.e. blue for cell tissues and red for TiO$_2$ agglomerates. At least five cells were analyzed to provide statistically significant results. As the sections of Fig. 4 show, the uptake of the TiO$_2$-NPs by the cells was mainly demonstrated for P25, food grade and platelet NPs (Fig. 4a,b,c) while no TiO$_2$ signal was registered inside the cells for bypiramids and rods (Fig. 4d,e).

4. Discussion

The aim of this study was to investigate the cytotoxicity and genotoxicity of three different shapes of TiO$_2$-NPs and to compare them with two commercially available TiO$_2$-NPs. The
factors taken into account for this study were: i) the physico-chemical properties of the particles such as shape, particle size, agglomeration state in culture media, crystalline phase, ii) the ability of the particles to induce cytotoxicity and genotoxicity, iii) the increase of the toxicological effects under light exposure due to the photocatalytic activity of TiO$_2$ and iv) the uptake of the NPs by human cells.

Published results on toxicity of TiO$_2$-NPs show high variability. Reasons for this variability include physico-chemical characteristics of NPs, different methods for prepare NPs dispersions, differences in NPs size and dispersion stability, and different exposure protocols (Charles et al., 2018). The characteristics of NPs dispersion can be influenced by medium components, such as serum proteins, and by NPs properties (size, shape, surface charge, surface coating etc.) (Huk et al. 2015). According to the study of Prasad et al. (2013), the present results showed that in all the TiO$_2$-NPs dispersions, the agglomeration state increases in base RPMI (without serum), while the size distribution in DMSO and in complete RPMI medium (with serum) is quite similar. The different agglomeration state is probably due to the ability of metal oxide NPs to adsorb proteins onto their surface, forming a “protein corona” which favors less agglomeration in complete medium, which contains more proteins (Prasad et al. 2013). Considering the results obtained, complete medium was selected as cytotoxicity/genotoxicity assay medium.

The viability of BEAS-2B treated with commercial and engineered TiO$_2$-NPs after exposure with light or in darkness was assessed using the WST-1 assay.

Commercial TiO$_2$-NPs induced low or no viability reduction (P25 and food grade, respectively) detected by WST-1 assay, such that these results are in agreement with some reports on the cytotoxicity of commercial TiO$_2$-NPs on BEAS-2B cells (Bhattacharya et al. 2009, Falck et al. 2009). Other studies showed that commercial TiO$_2$-NPs induced cytotoxicity on BEAS-2B (Shi et al. 2010, Ursini et al. 2014). According to the results on
P25, in the study of Prasad et al. (2013) 100 µg/ml of commercial P25 NPs produced a viability decrease in BEAS-2B cells after 24h exposure. In contrast, Park et al. (2008) found that exposure of BEAS-2B cells to commercial P25 (5-40 µg/ml) for 24h led to significant cell death, both in a time- and concentration-dependent manner. Instead, fewer studies have been performed using commercial food grade TiO$_2$-NPs. Proquin et al. (2017) tested these NPs on different cell lines: on Caco-2, they observed cytotoxicity, while, according to our results, on HCT116 they did not observe any cytotoxic effect up to the concentration of 100 µg/cm$^2$, confirming that the cytotoxicity can be influenced by the use of different cell lines.

Recently, the scientific community have produced reference NPs, which have been well characterized. About evaluation of these reference TiO$_2$-NPs, Di Bucchianico et al. (2016) assessed cytotoxic effects of some of these NPs (anatase 50-150 nm, anatase 5-8 nm, rutile 20-28 nm) in BEAS-2B cells; according to the present results the study shows in general no or low effects at the doses tested (2-100 µg/ml).

The data demonstrated that cytotoxicity was slightly affected by light exposure, which induced an increase of cellular damage after incubation with commercial P25 and engineered rods.

Comparing the results of WST-1 assay and LDH release, the first showed low cytotoxic effect at the doses tested, while the second did not show any cytotoxicity in both exposure protocols. The discrepancy between LDH release and WST-1 data suggests that the viability reduction may be caused by apoptosis, a cell death pathway in which the plasma membrane is maintained, as observed in other studies (Schilirò et al. 2015). This is in accordance with previous studies, which demonstrated that TiO$_2$-NPs could cause apoptosis in BEAS-2B cells (Park et al. 2008, Shi et al. 2010).
Results of Comet assay in presence of light and in darkness showed a significant DNA damage induced by commercial P25 and food grade NPs and engineered platelet NPs, while no genotoxicity was observed with other NPs.

Considering that the uptake of NPs could involve interactions of NPs with DNA, the observed genotoxic effect could be related to the presence of P25, food grade and platelet NPs into the BEAS-2B detected in the present study and by other studies (Bhattacharya et al. 2009, Park et al. 2008). The higher uptake of P25, food grade and platelet NPs could be explained with the higher agglomeration tendency (higher hydrodynamic diameter) (table 1).

Indeed, as supposed by Magdolenova et al. (2012b), TiO$_2$-NPs that form large agglomerates, differently from NPs that form smaller ones, precipitate at the bottom of the cell culture wells, increasing the real amount of NPs to which the cells are exposed. After penetration into the cells, these NPs may have direct access to DNA via transport into the nucleus and/or during mitosis when the dissolution of nuclear membrane occurs, so they could cause DNA breakage (Magdolenova et al. 2014). Other hypothesized mechanisms that can induce the observed DNA damage are that NPs (or metal ions released from particles) can enhance the permeability of the lysosomal membrane, inducing the release of DNases (Karlsson et al. 2010) or that NPs aggregates can deform nucleus causing DNA damage (Di Virgilio et al. 2010).

In addition, to quantify effects due to the photocatalytic activity of TiO$_2$, the highest doses (80, 120, 160 µg/ml) of NPs that showed a genotoxic effect were tested also in darkness (24h). Results showed that light exposure induced additional indirect genotoxicity, demonstrating a higher oxidative potential of TiO$_2$-NPs after exposure with light than in darkness. The presence of light increased DNA oxidative damage probably due to the photocatalytic activity of TiO$_2$-NPs, which caused an increase of NPs ability to produce radicals. In particular, based on previous studies, the anatase crystal structure of TiO$_2$ (used in
this study) seems to be the most catalytic/photocatalytic crystalline structure of TiO$_2$ and seems to be activated under both ultraviolet and visible light (Warheit and Donner 2015). A recent study (De Matteis et al. 2016) demonstrated that, in particular using anatase, light is a dominant factor to induce oxidative stress, TiO$_2$-NPs degradation and toxic effects. According to the present results, Gerloff et al. (2009) showed direct and oxidative genotoxic effects induced by TiO$_2$-NPs (80%/20% anatase-rutile) only in the presence of interior light. Karlsson et al. (2008) found that TiO$_2$-NPs (mixture of rutile and anatase) in darkness did not show oxidative DNA damage using the Fpg-Comet assay (Karlsson et al. 2010). However, as observed in the present study, also in darkness TiO$_2$-NPs can induce oxidative DNA damage, although lower than damage induced by light exposure. This result was observed also in the study of Gurr et al. (2005).

The results obtained highlight that food grade and engineered platelet NPs induced direct genotoxicity also in darkness. For the commercial food grade NPs the damage was lower than in presence of light. This result agree with the study of Gopalan et al. (2009); they suggest that TiO$_2$ (anatase 40 – 70 nm range) is capable of inducing higher direct genotoxic effects after simultaneous irradiation with UV, respect to genotoxicity induced in darkness. The increase of direct DNA damage after exposure with light, attested by Gopalan et al. (2009) and detected for food grade NPs, remain to be explained. A possible mechanisms, that may lead to this effect, could be related to the potential interaction of TiO$_2$-NPs with proteins involved in DNA repair, as demonstrated by Jugan et al. (2011). Genotoxicity is not only linked to the level of DNA damage but also to the type of lesions generated and their capacity to be repaired. NPs exposure in presence of light could influence activity of proteins such as repair enzymes, resulting in DNA damage not repaired or misrepaired (Magdolenova et al. 2014).
In conclusion, cytotoxicity of NPs was low, and was influenced by the NP shape as well as by light exposure. Instead, genotoxicity seemed to be influenced by the cellular-uptake and the aggregation tendency of TiO$_2$-NPs. These two aspects are probably related to different physico-chemical characteristics of NPs, such as the shape. Moreover, the presence of light enhanced the genotoxic effect of some NPs primarily increasing the oxidative stress. In summary, the results obtained indicate that the TiO$_2$-NPs shape may play a critical role in the potential genotoxicity and light can influence cytotoxicity and genotoxicity of both commercial and engineered TiO$_2$-NPs. The results obtained suggests that these shape engineered TiO$_2$-NPs are probably safer than the commercial ones.

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**Competing interests**

The authors declare that they have no competing interests.

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<table>
<thead>
<tr>
<th>Table</th>
<th>Sample</th>
<th>Particle size (nm)</th>
<th>$D_h$ DMSO (nm)</th>
<th>$D_h$ RPMI Base (nm)</th>
<th>$D_h$ RPMI Complete (nm)</th>
<th>Crystalline Phase</th>
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<tbody>
<tr>
<td>P25</td>
<td>20 ± 5</td>
<td>107 ± 31</td>
<td>722 ± 246</td>
<td>121 ± 37</td>
<td>Anatase:Rutile (5:1)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>quasi-spherical</td>
<td></td>
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<tr>
<td>Food grade</td>
<td>150 ± 50</td>
<td>184 ± 61</td>
<td>278 ± 54</td>
<td>184 ± 55</td>
<td>Anatase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>undefined shape</td>
<td></td>
<td></td>
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<tr>
<td>Bipyramids</td>
<td>50 ± 9</td>
<td>66 ± 20</td>
<td>259 ± 46</td>
<td>88 ± 24</td>
<td>Anatase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(aspect ratio 3:2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Rods</td>
<td>108 ± 47</td>
<td>36 ± 12</td>
<td>1500 ± 471</td>
<td>39 ± 17</td>
<td>Anatase</td>
</tr>
<tr>
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<tr>
<td></td>
<td>(aspect ratio 1:5)</td>
<td></td>
<td></td>
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<tr>
<td>Platelets</td>
<td>75 ± 25*</td>
<td>36 ± 10</td>
<td>233 ± 70</td>
<td>281 ± 83</td>
<td>250 ± 82</td>
<td>Anatase</td>
</tr>
<tr>
<td></td>
<td>(aspect ratio 8:1)</td>
<td></td>
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Table 1. Physico-chemical properties of the TiO$_2$-NPs samples. Data are presented as mean ± standard deviation of 500 NPs for the particle size and 5 measurements for the hydrodynamic diameter (D$_h$) of each sample. *The particle size was calculated along the major axis of the NPs.

**Figure captions**

Figure 1. SEM In-lens micrographs: (a) P25, (b) food grade, (c) bipyramids, (e) platelet NPs. T-SEM micrograph of rods (d). DLS analyses, normalized by volume distribution (f-j): (f) P25, (g) food grade, (h) bipyramids, (i) rods and (j) platelet NPs, suspensions in DMSO 1% (black line), RPMI base (red line) and RPMI complete (blue line).

Figure 2. Cytotoxicity (a,b) and lactate dehydrogenase (LDH) release (expressed as a percentage) (c,d) of BEAS-2B cells exposed to different concentrations (5–80 µg/ml) of commercial and engineered NPs. Control level is at 100%. Data represent effects detected
after exposure with light (a,c) and in darkness (b,d). Bars represent the mean %, error bars represent standard error of mean. *p<0.05; vs control cells (C-) according to ANOVA, followed by Dunnett’s test.

Figure 3. Effect of BEAS-2B cells exposure to commercial and engineered NPs evaluated by the Comet assay (±Fpg). Exposure with light (a-e): (a) P25, (b) food grade, (c) bipyramids, (d) rods, (e) platelet NPs; exposure in darkness (f-h): (f) P25, (g) food grade, (h) platelet NPs. Bars represent the mean % of tail intensity value, error bars represent standard error of mean. *p<0.05 vs control cells (C-). #p<0.05 treatment –Fpg vs treatment +Fpg. According to ANOVA, followed by Dunnett’s test.

Figure 4. 3D confocal micro-Raman imaging of BEAS-2B cells after exposure to commercial and engineered NPs. Top views (optical and 3D Raman) and 3D Raman sections are shown from the left to the right: (a) P25, (b) food grade, (c) platelet NPs, (d) bipyramids, (e) rods. 3D chemical images are built by superimposing the different maps of each cell at their corresponding focal planes and they are presented using a color meshwork i.e. blue for cell tissues (methylene blue ν(C-C)ring at 1600 cm⁻¹) and red for TiO₂ agglomerates (Eg band at 144 cm⁻¹ of the anatase TiO₂).
Figure 1
Figure 2

(a) Cell viability (%)
(b) Cell viability (%)
(c) LDH release (%)
(d) LDH release (%)

P25  Food grade  Bipyramids  Rods  Platelet NPs
Figure 3

(a) and (b) show the effects of varying concentrations of TiO$_2$ on tail intensity with and without Fpg treatment. (c) and (d) depict similar data but with different concentrations. (e) and (f) illustrate additional comparisons with different treatments. (g) and (h) provide further insights into the relationship between TiO$_2$ concentration and tail intensity under specific conditions.

The graphs indicate significant differences between the conditions, with marked changes observed in tail intensity as the TiO$_2$ concentration increases.