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**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1695126> since 2019-03-21T10:16:42Z

*Published version:*

DOI:10.1016/j.fct.2019.02.043

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1 **Shape-engineered titanium dioxide nanoparticles (TiO<sub>2</sub>-NPs): cytotoxicity and**  
2 **genotoxicity in bronchial epithelial cells**

3

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26 **List of Abbreviations**

27 BEAS-2B – Human bronchial epithelial cells

28  $D_h$  – Hydrodynamic diameter

29 Fpg – formamidopyrimidine glycosylase

30 NP – Nanoparticle

31  $TiO_2$  – Titanium dioxide

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51 **Abstract**

52 The aim of this study was to evaluate cytotoxicity (WST-1 assay), LDH release (LDH assay)  
53 and genotoxicity (Comet assay) of three engineered TiO<sub>2</sub>-NPs with different shapes  
54 (bipyramids, rods, platelets) in comparison with two commercial TiO<sub>2</sub>-NPs (P25, food grade).  
55 After NPs characterization (SEM/T-SEM and DLS), biological effects of NPs were assessed  
56 by exposing BEAS-2B cells in the presence of light and in the absence. The cellular uptake of  
57 NPs was analyzed using Raman spectroscopy.

58 The cytotoxic effects were mostly slight. After light exposure, using the WST-1 assay, the  
59 largest cytotoxicity was observed for rods; P25, bipyramids and platelets showed a similar  
60 effect, while no effect was induced by food grade. No LDH release was detected using the  
61 LDH assay, confirming the low effect on plasma membrane. Regarding genotoxicity, food  
62 grade and platelets induced direct genotoxic effect while P25, food grade and platelets caused  
63 oxidative DNA damage. No genotoxic or oxidative DNA damage was induced by bipyramids  
64 and rods. In darkness biological effects were overall lower than after light exposure.  
65 Considering that only food grade, P25 and platelets (more agglomerated) were internalized by  
66 cells, the uptake resulted correlated with genotoxicity.

67 In conclusion, cytotoxicity of NPs was low, influenced by shape as well as by light exposure.  
68 Instead, genotoxicity seemed to be influenced by cellular-uptake and aggregation tendency.

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70 **Keywords:** shape-engineered TiO<sub>2</sub> nanoparticles; genotoxic and oxidative damage; Comet  
71 assay; cytotoxicity; Raman spectroscopy.

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## 76 **1. Introduction**

77 Nanoparticles (NPs) are defined as particles having their three dimension in the range of 1 –  
78 100 nm (ISO 2015). Actually, many consumer products incorporates NPs. The technological,  
79 medical and economic benefits of NPs are considerable, but the presence of nanoparticles in  
80 the environment could cause adverse effects to humans. NPs have a greater surface area per  
81 mass unit, so they potentially have an increased biological activity compared to fine particles.  
82 Moreover, NPs size is comparable to the size of cellular structures, so NPs might potentially  
83 emulate biological molecules or interfere physically with biological processes (Magdolenova  
84 et al. 2012a).

85 TiO<sub>2</sub> is the oxide of titanium and it has different crystalline structures: anatase, brookite and  
86 rutile. Brookite is not produced by industry and is not incorporated in commercial products. In  
87 contrast, rutile and anatase are largely used in commercial products (Jovanovic 2015). TiO<sub>2</sub> is  
88 one of the most frequently applied NPs and it is in the top five NPs used in consumer products  
89 (Shi et al. 2013). TiO<sub>2</sub>-NPs produced are used primarily as a pigment owing to their brightness,  
90 resistance to discoloration and high refractive index. As a pigment, TiO<sub>2</sub>-NPs are incorporated  
91 in paints, plastic materials, paper, foods, medical products and cosmetics. Due to its catalytic  
92 and photocatalytic properties, TiO<sub>2</sub> is also used as an antimicrobial agent and a catalyst for  
93 purification of air and water (Bonetta et al. 2013, Tomankova et al. 2015).

94 TiO<sub>2</sub>-NPs could be engineered in terms of shapes and sizes by changing synthesis conditions  
95 such as raw material, temperature, acidic and alkaline conditions. Engineered TiO<sub>2</sub>-NPs with  
96 various shapes (e.g. rods, dots and belts) have been prepared for different applications (Bernard  
97 and Curtiss 2005, Sha et al. 2015, Wang et al. 2004). In particular engineered fiber-shaped  
98 nanomaterials (i.e. nanowires, nanotubes) are very attractive because they showed higher  
99 activity and advantages in photocatalysis, charge transfer and sensing applications due to their

100 structure (Hamilton et al. 2009). However, these new and enhanced properties may also induce  
101 higher toxicological effects upon exposure with biological tissues.

102 Humans can be exposed to TiO<sub>2</sub>-NPs via three portals of entry: oral (mainly via food  
103 consumption), dermal (often through cosmetic and sunscreen applications) and inhalation  
104 (mainly under occupational and manufacturing conditions) (Warheit and Donner 2015).

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

112 Many *in vitro* studies showed cytotoxicity, genotoxicity and oxidative effects induced by TiO<sub>2</sub>-  
113 NPs through oxidants generation, inflammation and apoptosis (Jugan et al. 2011, Karlsson et  
114 al. 2015, Park et al. 2008, Shi et al. 2010). The potential of NPs to cause DNA damage is an  
115 important aspect that needs attention due to possible mutations and carcinogenesis. Physico-  
116 chemical characteristics of NPs have an important role in toxicity. Different studies showed  
117 that biological effects can be influenced by crystalline structure, size, shape, exterior area,  
118 agglomeration/aggregation and surface properties (Bhattacharya et al. 2009, Johnston et al.  
119 2009). Some studies revealed that crystalline structure probably influences the induced toxicity,  
120 in particular the anatase seems to be more reactive (Sayes et al. 2006) and induces more toxic,  
121 genotoxic and inflammatory effects, than the rutile (Falck et al. 2009, Petkovic et al. 2011, Xue  
122 et al. 2010). However, other studies gave contradictory results with rutile forms being more  
123 toxic than anatase (Gurr et al. 2005, Numano et al. 2014, Uboldi et al. 2016). The effect of  
124 agglomeration/aggregation of NPs on toxicity is not well understood yet. In recent studies,

125 some authors demonstrated that agglomeration can influence NPs genotoxicity (Magdolenova  
126 et al. 2012b, Prasad et al. 2013).

127 Although physico-chemical properties of NPs can have an important role in the impact on their  
128 toxicity, only few studies on shape dependent TiO<sub>2</sub> toxicity has been conducted (Allegri et al.  
129 2016, Hamilton et al. 2009, Park et al. 2013). Additional studies are needed to evaluate the role  
130 of shape on TiO<sub>2</sub>-NPs toxicity in order to produce useful data for assessing the safety of  
131 engineered NPs.

132 To address this issue, the aim of this study was to investigate cytotoxicity (WST-1 assay), LDH  
133 release (LDH assay) and genotoxicity (Comet assay) of three types of engineered TiO<sub>2</sub>-NPs  
134 of different shapes (bipyramids, rods and platelet NPs) in BEAS-2B (cells isolated from human  
135 bronchial epithelium) in comparison with two commercial types of TiO<sub>2</sub>-NPs (P25 and food  
136 grade). Since the exposure to TiO<sub>2</sub>-NPs mainly occurs through respiratory tract (occupational  
137 and manufacturing conditions), human cells of the respiratory system (such as BEAS-2B), were  
138 selected as a good cell model for *in vitro* toxicology tests. All the TiO<sub>2</sub>-NPs in this study were  
139 first physico-chemically characterized, even in different culture media to study their  
140 agglomeration state, and then they were biologically evaluated. In order to take into account  
141 the photocatalytic properties of the TiO<sub>2</sub>-NPs, we investigated the cytotoxicity and  
142 genotoxicity on BEAS-2B under light exposure and in darkness. Moreover, a modern  
143 application of Raman spectroscopy, the 3D confocal Raman imaging, was used to study the  
144 uptake of the NPs within the BEAS-2B cells, as the Raman spectra provide information about  
145 both organic molecules and solid NPs simultaneously (Ahlinder et al. 2013).

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150 **2. Materials and methods**

151 **2.1 Synthesis and Preparation of TiO<sub>2</sub> NPs dispersion**

152 Rods and bipyramids TiO<sub>2</sub>-NPs were synthesized by the forced hydrolysis of an aqueous  
153 solution of TiIV(triethanolamine)<sub>2</sub>titanatranne (Ti(TEOAH)<sub>2</sub>), using triethanolamine (TEOA)  
154 as shape controller; pH of synthesis was adjusted by adding 1 M NaOH solution; details of  
155 these procedures were previously reported (Iannarelli et al. 2016, Lavric et al. 2017). The  
156 synthesis of platelet NPs was performed with a solvothermal method (Han et al. 2009, Zhang  
157 et al. 2012). In a typical synthesis: a precise volume of Ti(OBu)<sub>4</sub> was added in a 150 ml Teflon  
158 pot and the desired volume of concentrated hydrofluoric acid was added dropwise under  
159 stirring. The Teflon pot was sealed and kept under stirring at high temperature (250°C) for 24h  
160 in autoclave. The resulting paste was centrifuged three times and washed with acetone and with  
161 water (Milli-Q) to remove the residual organics. The synthesis dispersions were subjected to  
162 dialysis process (against ultrapure water, using Spectra/Por dialysis membrane tubing MWCO  
163 8–14 kDa) in order to clean the medium. To avoid agglomeration and precipitation,  
164 dimethylsulfoxide (DMSO 1% in water) was added to the NPs dispersions (final concentration  
165 2.5 mg/ml); the dispersions were homogenized using an ultra-sonication procedure (Iannarelli  
166 et al. 2016), few hours before the exposure with cells.

167 The same procedure was employed in the preparation of the dispersion of commercial TiO<sub>2</sub>  
168 powders, which were the P25 NPs (Evonik), extensively used in toxicity studies (Karlsson et  
169 al. 2015, Magdolenova et al. 2014, Valant et al. 2012), and the food grade NPs (Faravelli  
170 Group), incorporated in many edible products (Weir et al. 2012).

171 **2.2 Scanning Electron Microscopy (SEM) including Transmission Mode (T-SEM)**

172 The dimensional characterization (size and shape) of TiO<sub>2</sub>-NPs was carried out with SEM using  
173 a Zeiss Supra 40 instrument (Zeiss) equipped with a Schottky field emitter, the standard  
174 secondary electrons, i.e. Everhart-Thornley, detector and a high-resolution In-lens detector.



175 The surface-sensitive In-lens SEM mode better suited to morphological/shape analysis and  
176 transmission mode in SEM (T-SEM) better suited for dimensional measurements were applied  
177 complementary to the same field of view on the sample.

### 178 ***2.3 Dynamic Light Scattering (DLS) analysis***

179 Delsa Nano™ C Analyzer (Beckman Coulter) equipped with a 638 nm diode laser and a  
180 temperature control was used for the DLS measurements. The laser fluctuation was detected  
181 on a photomultiplier tube detector positioned behind the cuvette with an angle of 163°.  
182 Hydrodynamic diameters were calculated setting temperature at 25°C, viscosity ( $\eta$ ) 0.890 cP  
183 and refractive index of water 1.3325. In order to simulate the culture medium conditions, DLS  
184 analyses were conducted on dilution of TiO<sub>2</sub> dispersions (1:4) in a 1% DMSO aqueous solution,  
185 as reference analysis, and in base RPMI 1640 medium [supplemented with l-glutamine (4 mM)  
186 and penicillin-streptomycin (100 U/ml - 100  $\mu$ g/ml)] and complete RPMI 1640 medium  
187 [supplemented with FBS (10% v/v), l-glutamine (4 mM) and penicillin-streptomycin (100  
188 U/ml - 100  $\mu$ g/ml)].

### 189 ***2.4 Raman spectroscopy analysis***

190 The aqueous suspensions of the TiO<sub>2</sub>-NPs under investigation were freeze-dried to obtain a  
191 solid powder. Raman spectroscopy was used in the analysis of dry TiO<sub>2</sub>-NPs powder using a  
192 DXR™ Raman Microscope (Thermo Scientific) with a laser wavelength at 532 nm, a laser  
193 power of 1 mW and a 10x microscope objective. Spectra were collected in the 50–1800 cm<sup>-1</sup>  
194 spectral region, with a grating resolution of 3.3–3.9 cm<sup>-1</sup>, exposure time of 1 s and 20 scans in  
195 total.

### 196 ***2.5 Cell culture and exposure***

197 BEAS-2B cells, isolated from human bronchial epithelium, were obtained from the American  
198 Type Culture Collection (ATCC® CRL-9609™). BEAS-2B were grown as a monolayer,  
199 maintained and treated in complete RPMI 1640 medium [supplemented with FBS (10% v/v),

200 l-glutamine (4 mM) and penicillin-streptomycin (100 U/ml - 100 µg/ml)], at 37°C in a  
201 humidified atmosphere containing 5% CO<sub>2</sub>.

202 The solution of NPs (2.5 mg/ml, DMSO 1% in water) was vortexed and sonicated (30 min) in  
203 order to homogenize the NPs. NPs (5 – 160 µg/ml) were directly pipetted in culture plates  
204 containing RPMI 1640 medium and then the cell culture plates were mixed on a shaker (10  
205 min). The cells were exposed for 1h under laboratory light and then incubated at 37 °C in  
206 darkness (23h) (exposure with light). In order to standardize the exposure with light the cells  
207 were exposed in a dark room (obscured by daylight) to a normal laboratory lamp (36W/840  
208 Lumilux Cool White-36 W, 3350 lm, 4000 K-supplied from OSRAM lighting AG). The lamp  
209 illuminance measured with Quantum photo/radiometer HD 9021 (Delta Ohm) was 289±11 lx.  
210 To quantify effects due to the photocatalytic activity of TiO<sub>2</sub>, cells were exposed for 24h in  
211 darkness (exposure in darkness).

212 After exposure, cytotoxicity and genotoxicity assays were performed.

## 213 **2.6 Cytotoxicity**

214 Cell viability was assessed using Cell Proliferation Reagent WST-1 (Roche). The assay was  
215 performed as previously described by Gea et al. (2018). Briefly, BEAS-2B cells were cultured  
216 in 75 cm<sup>2</sup> flasks and maintained until the cells reached 70 % confluence; cells were then seeded  
217 in 24-well plates (5×10<sup>4</sup> cells/well) and allowed to adhere overnight. After that, culture medium  
218 was removed and cells were exposed to NPs (5, 10, 20, 50 and 80 µg/ml, equivalent to 1.3, 2.6,  
219 5.2, 13.0, 20.7 µg/cm<sup>2</sup>) for 24h with light or in darkness (as specified in paragraph 2.5). After  
220 exposure, WST-1 was added (50 µl/well) and incubated for 3h (37 °C). After incubation, well  
221 contents were centrifuged and the supernatants were transferred in 96-well plate to remove the  
222 interference owing to the NPs. The absorbance was measured at 440 nm (Tecan Infinite Reader  
223 M200 Pro). Absorbance of unexposed cells was used as negative control. Data were expressed

224 as a percentage of viability. All experiments were performed in quadruplicate (four wells for  
225 each experimental condition).

226 As indicator of cell membrane damage, lactate dehydrogenase activity was measured in cell-  
227 free culture supernatants using the LDH assay kit (Cytotoxicity Detection Kit PLUS, Roche)  
228 modified for NPs exposure. Briefly, BEAS-2B cells were cultured in 75 cm<sup>2</sup> flasks and  
229 maintained until the cells reached 70 % confluence, cells were then seeded in 24-well plates (5  
230 × 10<sup>4</sup> cells/well) and allowed to adhere overnight. After that, culture medium was removed and  
231 the cells were exposed to NPs (5, 10, 20, 50 and 80 µg/ml, equivalent to 1.3, 2.6, 5.2, 13.0,  
232 20.7 µg/cm<sup>2</sup>) for 24h with light or in darkness (as specified in paragraph 2.5). After exposure,  
233 the contents of each well were centrifuged to remove the interference owing to the NPs. Each  
234 supernatant (100 µl) was transferred into 96-well plate, mixed with Reaction Mixture (100  
235 µl/well) and incubated for 30 min at 15 – 25 °C. After incubation, Stop Solution (50 µl/well)  
236 was added and the absorbance was measured at 490 nm (Tecan Infinite Reader M200 Pro).  
237 Absorbance measurement of unexposed cells were used as negative control, while absorbance  
238 measurement of unexposed cells lysed with Lysis Solution (Cytotoxicity Detection Kit PLUS,  
239 Roche) was used as positive control. Data were expressed as a percentage of LDH release,  
240 respect to control cells (100%). All experiments were performed in triplicate (three wells for  
241 each experimental condition).

## 242 **2.7 Genotoxicity**

243 The alkaline Comet assay was used for DNA damage evaluation (direct DNA damage). BEAS-  
244 2B cells were cultured in 75 cm<sup>2</sup> flasks and maintained until the cells reached 70 % confluence;  
245 cells were then seeded in 6-well plates (3 × 10<sup>5</sup> cells/well) and cultured overnight before  
246 exposure to NPs. The cells were exposed to different doses of NPs (20, 50, 80, 120 and 160  
247 µg/ml, equivalent to 5.2, 13.0, 20.8, 31.2, 41.6 µg/cm<sup>2</sup>) for 24h with light or in darkness (as  
248 specified in paragraph 2.5). Unexposed cells and cells treated with DMSO (1%) were used as

249 negative controls. The alkaline Comet assay was performed according to Tice et al. (2000) after  
250 slight modifications (Bonetta et al. 2018). After exposure, cells were washed with base RPMI  
251 1640 and PBS, detached using trypsin-EDTA (1x) and cell viability was determined (trypan  
252 blue staining). Cells were then centrifuged and mixed with low melting point agarose (0.7%),  
253 placed on the slides coated with normal melting agarose (1%) and low melting point agarose  
254 added as the top layer. The slides were immersed in lysis solution in the dark overnight (8 mM  
255 Tris-HCl, 2.5 M NaCl, 100 mM EDTA disodium salt dihydrate, 1% TRITON X-100 and 10%  
256 DMSO, pH 10, 4°C). For the unwinding, the slides were immersed in alkaline electrophoresis  
257 buffer (20 min) (1 mM EDTA, 300 mM NaOH, 10% DMSO, pH > 13) and the electrophoresis  
258 was carried out in the same buffer (20 min, 1 V/cm and 300 mA). The slides were washed with  
259 neutralization buffer (0.4 M Tris-HCl, pH 7.5, 4 °C, 3 min), fixed with ethanol 70% (-20 °C, 5  
260 min) and air dried. All steps were performed under yellow light to prevent additional DNA  
261 damage. Slides were stained with ethidium bromide (20 µg/ml) and analyzed using a  
262 fluorescence microscope (Axioskop HBO 50, Zeiss). The percentage of tail intensity was used  
263 to estimate DNA damage. A total of a hundred randomly selected cells per treatment (two gels  
264 per slides) were analyzed using the Comet Assay IV software (Perceptive Instruments, Instem).  
265 Two independent experiments were performed for each experimental condition.

266 Genotoxic effect (direct DNA damage) was evaluated comparing cells exposed to NPs with  
267 control cells (DMSO 1%).

268 The formamidopyrimidine glycosylase (Fpg)-modified Comet assay was performed for DNA  
269 damage evaluation (direct + indirect DNA damage) as reported in Bonetta et al. (2009) with  
270 slight modification (Gea et al. 2018). The test was carried out as described for the alkaline  
271 Comet assay but, after lysis, the slides were washed with Fpg Buffer (5 min for three times)  
272 (40 mM Hepes, 0.1M KCl, 0.5 mM EDTA disodium salt dihydrate, 0.2 mg/ml bovine serum

273 albumin, pH 8). Then, each gel was incubated with 1 unit of Fpg enzyme (*Escherichia coli*)  
274 (TREVIGEN) at 37°C for 30 min. Procedure control slides were incubated with buffer only.  
275 Cells treated with DMSO (1%) and enzyme were used as negative controls. Two independent  
276 experiments were performed for each experimental condition.

277 The DNA damage (direct + indirect DNA damage) was evaluated comparing cells exposed to  
278 NPs with control cells (DMSO 1% +Fpg).

279 The oxidative damage was calculated subtracting the mean tail intensity (%) in enzyme-treated  
280 cells (+Fpg) from the relative mean tail intensity (%) in enzyme-untreated cells (-Fpg).

### 281 **2.8 3D confocal micro-Raman imaging spectroscopy**

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

293 3D confocal micro-Raman imaging spectroscopy of BEAS-2B cells was conducted with a  
294 DXR™xi Raman Imaging Microscope (Thermo Scientific) using a laser wavelength at 532  
295 nm, a 1 mW laser power, a 100X microscope objective and a motorized stage with a 1 µm of  
296 step size and a 1 µm offset. Spectra were collected in the 50–3500 cm<sup>-1</sup> spectral region with a  
297 grating resolution of 5 cm<sup>-1</sup>, an exposure time of 0.025 s and 5 scans in total. 3D Raman images

298 were reconstructed taking the Raman peaks at  $1600\text{ cm}^{-1}$  of methylene blue and the  $E_g$  band at  
299  $144\text{ cm}^{-1}$  of the  $\text{TiO}_2$ -NPs, respectively. Each cell was investigated at different focal planes and  
300 a chemical image was obtained by the combination of the  $\nu(\text{C-C})$  ring at  $1600\text{ cm}^{-1}$  of the  
301 methylene blue and the  $E_g$  band at  $144\text{ cm}^{-1}$  of the  $\text{TiO}_2$ -NPs. Since methylene blue is contained  
302 in the Giemsa stain and it is widely distributed into the fixed cells, its signals were considered  
303 representative of the entire volume of the cells. As far as the tracking of the NPs are concerned,  
304 the  $E_g$  band at  $143\text{ cm}^{-1}$  is the most intense signal in the molecular fingerprint of the anatase  
305  $\text{TiO}_2$  and the region between  $50\text{ cm}^{-1}$  and  $400\text{ cm}^{-1}$  in the Raman spectrum is usually free of  
306 the vibrational bands of biological species. Therefore, this signal was selected to sensitively  
307 locate the  $\text{TiO}_2$ -NPs inside the cells. Image J software was used in the development of the 3D  
308 chemical images both for cells and  $\text{TiO}_2$ -NPs, which were superimposed using a Solidworks®  
309 2016 Cad based software. 3D Raman chemical images are presented using a color meshwork  
310 i.e. blue for cell tissues and red for  $\text{TiO}_2$  agglomerates.

## 311 **2.9 Statistical analysis**

312 IBM SPSS software (ver. 24.0) was used to perform statistical analysis. The results of WST-  
313 1, LDH and Comet assays are presented as the mean  $\pm$  standard deviation. Differences between  
314 exposed and control cells were tested by ANOVA followed by the post hoc Dunnett's test  
315 procedure. Differences between light and dark exposure were tested by ANOVA, followed by  
316 the post hoc Tukey's test procedure. Data were considered statistically different for a p-value  
317 less than 0.05.

318

## 319 **3. Results**

### 320 **3.1 Raman characterization of NPs and size distribution**

321 In order to establish a relationship among the physico-chemical features of NPs and their ability  
322 to induce a toxic effect, well-defined and controlled protocols were developed for the

323 production of engineered anatase TiO<sub>2</sub>-NPs with different shapes. All the NPs produced in this  
324 study were first characterized with a SEM equipped with a transmission-unit for T-SEM, which  
325 provided information both on the shape and the size of the constituent NPs (Fig. 1a-e). The Fig.  
326 1 and Table 1 show shapes and particle size of commercial TiO<sub>2</sub>-NPs and fabricated engineered  
327 TiO<sub>2</sub>-NPs.

328 These NPs were also characterized by Dynamic Light Scattering (DLS) as a quick method for  
329 sizing and determining the state of NP agglomeration. For each kind of sample, the  
330 agglomeration in 1% DMSO aqueous solution, in base RPMI [supplemented with l-glutamine  
331 (4 mM) and penicillin-streptomycin (100 U/ml - 100 µg/ml)] and complete RPMI  
332 [supplemented with FBS (10% v/v), l-glutamine (4 mM) and penicillin-streptomycin (100  
333 U/ml - 100 µg/ml)] (Fig. 1f-j) were compared. In all the TiO<sub>2</sub> materials considered for this  
334 study, the agglomeration state increase in base RPMI, while the size distribution in DMSO and  
335 in complete RPMI is quite similar.

336 The crystalline composition of the TiO<sub>2</sub>-NPs, analyzed by Raman spectroscopy, showed a  
337 typical fingerprint of the anatase TiO<sub>2</sub> (Fig. S.1) with the characteristic phonon bands E<sub>g</sub> at  
338 143 cm<sup>-1</sup>, E<sub>g</sub> at 197 cm<sup>-1</sup>, A<sub>1g</sub> at 397 cm<sup>-1</sup>, B<sub>1g</sub> at 515 cm<sup>-1</sup> and E<sub>g</sub> at 639 cm<sup>-1</sup> for all the  
339 investigated NPs. Since P25 is a known mixture of anatase and rutile (5:1), with also a small  
340 amount of amorphous TiO<sub>2</sub> (Ohtani et al. 2010), its Raman spectrum still retains all the typical  
341 anatase Raman bands but it also contains two small shoulders at 450 cm<sup>-1</sup> and 600 cm<sup>-1</sup>, which  
342 were assigned to the E<sub>g</sub> and A<sub>1g</sub> phonon bands, respectively, of rutile (Tompsett et al. 1995).

343 All the physiochemical properties of the TiO<sub>2</sub>-NPs under study such as shape, particle size,  
344 hydrodynamic diameter in different liquid media and the crystalline phase are summarized in  
345 Table 1.

### 346 **3.2 Cytotoxicity**

347 The results of the effects of different TiO<sub>2</sub>-NPs concentration on cell viability (WST-1 assay)  
348 are reported in Fig. 2a (exposure with light) and in Fig. 2b (exposure in darkness).  
349 In general, a low cytotoxic effect was observed at the tested doses both in the exposure with  
350 light and in the exposure in darkness. The observed viability ranged from 102.8 to 88.4% for  
351 the exposure with light and from 99.6 to 87.4% for the exposure in darkness.  
352 Considering the exposure with light, the commercial P25 induced a slight decrease in viability  
353 starting from the doses of 50 µg/ml ( $p<0.05$ ) while no cytotoxic effects were observed for the  
354 other commercial NPs (food grade) at the tested concentrations. As far as engineered NPs are  
355 concerned, bipyramids and platelet NPs induced the same cytotoxic effect of commercial P25  
356 NPs; on the contrary, rods is the NP shape with higher cytotoxic effect showing a viability  
357 decrease already starting from 10 µg/ml ( $p<0.05$  or  $p<0.001$ ).  
358 Considering the exposure in darkness, a lower cytotoxic effect was observed for commercial  
359 P25 NPs with respect to light exposure because a slight decrease in viability was observed for  
360 P25 NPs only at the highest dose (80 µg/ml) ( $p<0.05$ ). As reported after exposure with light,  
361 no cytotoxic effect was observed for the other commercial NPs (food grade). About engineered  
362 NPs, the exposure in darkness did not modify the cytotoxic effect of bipyramids NPs resulting  
363 in a viability reduction starting from the dose of 50 µg/ml ( $p<0.001$ ) as reported in the  
364 experiment with light. In contrast, in the darkness, rods NPs showed a lower cytotoxic effect  
365 than observed with light because a slight decrease in viability was observed for rod NPs only  
366 starting from the dose of 20 µg/ml ( $p<0.05$ ). As during the exposure with light, platelet NPs  
367 induced a decrease in viability; the cytotoxic effect was significant starting from a less dose  
368 (10 µg/ml,  $p<0.05$ ) than in the experiment with light (50 µg/ml).  
369 The results of the effects of different TiO<sub>2</sub>-NPs concentration on LDH release has been reported  
370 in Fig. 2c (exposure with light) and in Fig. 2d (exposure in darkness).



371 No significant LDH release was detected using LDH assay in both exposure protocols (with  
372 light or in darkness), confirming the low cytotoxic effect evidenced by WST-1 assay.

### 373 **3.3 Genotoxicity**

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

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

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

394 As demonstrated by other authors (Kalsson 2010, Karlsson et al. 2015), an interference during  
395 the scoring of the assay was detected in particular at the higher doses of P25 and platelet NPs,

396 indeed nanoparticles with some autofluorescence were visible in the comets “head” and the  
397 stained DNA appeared faded. The interference probably caused the loss of concentration-  
398 dependent increase in DNA direct and oxidative damage observed for the higher doses. The  
399 phenomenon could be explained also considering that base oxidation is hard to measure  
400 accurately when there are a lot of strand breaks, because the Comet assay becomes saturated  
401 (Collins et al. 2017).

402 In order to evaluate the role of the light on the genotoxic and oxidative damage induced by  
403 commercial and engineered NPs, the highest doses (80, 120, 160  $\mu\text{g/ml}$ ) of NPs that showed a  
404 genotoxic effect (P25, food grade and platelet NPs) were tested in darkness (24h).

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

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

### 421 **3.4 Confocal micro-Raman spectroscopy**

422 The confocal micro-Raman imaging spectroscopy was used in order to evaluate qualitatively  
423 the presence/absence of different types of TiO<sub>2</sub>-NPs inside the cells. 3D chemical images are  
424 built by superimposing the different maps of each cell at their corresponding focal planes and  
425 they are presented using a color meshwork i.e. blue for cell tissues and red for TiO<sub>2</sub>  
426 agglomerates. At least five cells were analyzed to provide statistically significant results. As  
427 the sections of Fig. 4 show, the uptake of the TiO<sub>2</sub>-NPs by the cells was mainly demonstrated  
428 for P25, food grade and platelet NPs (Fig. 4a,b,c) while no TiO<sub>2</sub> signal was registered inside  
429 the cells for bypiramids and rods (Fig. 4d,e).

430

### 431 **4. Discussion**

432 Many *in vitro* studies have been conducted to investigate cytotoxicity/genotoxicity of TiO<sub>2</sub>-  
433 NPs but the results are often conflicting and employed doses were sometimes high (Valant et  
434 al., 2012; Chen et al., 2014; Magdolenova et al., 2014; Karlsson et al., 2015; Moller et al.,  
435 2015a). The aim of this study was to investigate the cytotoxicity and genotoxicity of three  
436 different shapes of TiO<sub>2</sub>-NPs and to compare them with two commercially available TiO<sub>2</sub>-NPs.  
437 The issues taken into account for this study were: i) the physico-chemical properties of the  
438 particles (shape, particle size, agglomeration state in culture media, crystalline phase) that can  
439 influence biological effects, ii) the ability of the particles to induce cytotoxicity and  
440 genotoxicity, iii) the increase of the toxicological effects under light exposure due to the  
441 photocatalytic activity of TiO<sub>2</sub> and iv) the uptake of the NPs by human cells.

442 In the present study, the cytotoxicity assays were selected in order to reduce the interference of  
443 NPs with the assays (interference with optical detection methods, ability to convert the  
444 substrates). Moreover, as suggested by other studies (Wilhelmi et al., 2012; Guadagnini et al.,  
445 2015; Popescu et al., 2015), the assays processes were optimized for evaluation of NPs and

446 (before the assessment of NPs cytotoxicity) relevant controls, assessing particles for their  
447 ability to interfere with the assays, were conducted (data not shown).

448 For genotoxicity evaluation, a literature revision (on application of genotoxicity assays testing  
449 NPs) was made before the application of Comet assay (Karlsson 2010; Magdolenova et al.,  
450 2012a; Karlsson et al., 2015; Cowie et al., 2015; Moller et al., 2015b; Huk et al., 2015). To  
451 ensure the correct evaluation of DNA damage two independent experiment were performed for  
452 each experimental condition. The analysis of each comet was made using the Comet Assay IV  
453 and the automatic evaluation proposed by the software was carefully checked by an operator.

454 Published results on toxicity of TiO<sub>2</sub>-NPs show high variability. Reasons for this variability  
455 include physico-chemical characteristics of NPs, different methods to prepare NPs dispersions,  
456 differences in NPs size and dispersion stability, and different exposure protocols (Charles et  
457 al., 2018). The characteristics of NPs dispersion can be influenced by medium components,  
458 such as serum proteins, and by NPs properties (size, shape, surface charge, surface coating etc.)  
459 (Huk et al. 2015). According to the study of Prasad et al. (2013), the present results showed  
460 that in all the TiO<sub>2</sub>-NPs dispersions, the agglomeration state increases in base RPMI (without  
461 serum), while the size distribution in DMSO and in complete RPMI medium (with serum) is  
462 quite similar. The different agglomeration state is probably due to the ability of metal oxide  
463 NPs to adsorb proteins onto their surface, forming a “protein corona” which favors less  
464 agglomeration in complete medium, which contains more proteins (Prasad et al. 2013).  
465 Considering the results obtained, complete medium was selected as cytotoxicity/genotoxicity  
466 assay medium.

467 The viability of BEAS-2B treated with commercial and engineered TiO<sub>2</sub>-NPs after exposure  
468 with light or in darkness was assessed using the WST-1 assay.

469 Commercial TiO<sub>2</sub>-NPs induced low (P25) or no viability reduction (food grade) detected by  
470 WST-1 assay; these results are in agreement with some reports on commercial TiO<sub>2</sub>-NPs

471 (Bhattacharya et al. 2009, Falck et al. 2009). Previous studies that investigated the cytotoxicity  
472 of commercial P25 on BEAS-2B showed that only 100 µg/ml of commercial P25 NPs produced  
473 a viability decrease after 24h exposure (Prasad et al. 2013). Fewer studies have been performed  
474 using commercial food grade TiO<sub>2</sub>-NPs. Proquin et al. (2017) tested these NPs on different cell  
475 lines: on Caco-2, they observed cytotoxicity, while on HCT116 they did not observe any  
476 cytotoxic effect up to the concentration of 100 µg/cm<sup>2</sup>. The result obtained on HCT116 was in  
477 accordance with the low cytotoxic effect induced by commercial food grade TiO<sub>2</sub>-NPs detected  
478 in the present study. Recently, the scientific community have produced reference NPs, which  
479 have been well characterized. Di Bucchianico et al. (2016) assessed cytotoxic effects of some  
480 of these NPs (anatase 50-150 nm, anatase 5-8 nm, rutile 20-28 nm) in BEAS-2B cells and,  
481 according to the present results, showed in general no or low effects at the tested doses (2-100  
482 µg/ml).

483 On the contrary, other studies showed that commercial TiO<sub>2</sub>-NPs induced higher cytotoxicity  
484 on BEAS-2B (Shi et al. 2010, Ursini et al. 2014). In particular, Park et al. (2008) found that  
485 exposure of BEAS-2B cells to commercial P25 (5-40 µg/ml) for 24h led to significant cell  
486 death, both in a time- and concentration-dependent manner.

487 The data of present study demonstrated that cytotoxicity was slightly affected by light  
488 exposure, which induced an increase of cellular damage after incubation with commercial P25  
489 and engineered rods. The influence of light exposure on cytotoxicity was also observed in other  
490 studies (Vevers and Jha 2008, Reeves et al. 2008). Differently from P25 and rods, exposure to  
491 platelet NPs induced higher cytotoxicity in darkness than after light exposure; the mechanism  
492 that led to this result is not clear.

493 Comparing the results of cytotoxicity (WST-1 assay) and LDH release, the first showed low  
494 cytotoxic effect at the doses tested, while the second did not show any cytotoxicity in both  
495 exposure protocols. The discrepancy between cytotoxicity (WST-1) and LDH release data

496 suggests that the viability reduction may be caused by apoptosis, a cell death pathway in which  
497 the plasma membrane is maintained, as observed in other studies (Schilirò et al. 2015). This is  
498 in accordance with previous studies, which demonstrated that TiO<sub>2</sub>-NPs could cause apoptosis  
499 in BEAS-2B cells (Park et al. 2008, Shi et al. 2010). The observed discrepancy could be also  
500 explained considering that the tested compounds (TiO<sub>2</sub>-NPs) could induce an effect on the  
501 intracellular activity (mitochondria activity) without causing plasma membrane breakage, as  
502 observed by other authors (Weyermann et al. 2005, Fotakis and Timbrell 2006).

503 Results of Comet assay in presence of light and in darkness showed a significant DNA damage  
504 induced by commercial P25 and food grade NPs and engineered platelet NPs, while no  
505 genotoxicity was observed with the other engineered NPs (bipyramids and rods).

506 Considering that the uptake of NPs could involve interactions of NPs with DNA, the observed  
507 genotoxic effect could be related to the presence of P25, food grade and platelet NPs into the  
508 BEAS-2B as observed by other authors (Bhattacharya et al. 2009, Park et al. 2008).

509 In the present study, the higher uptake of P25, food grade and platelet NPs seemed to be related  
510 with higher agglomeration tendency (higher measure of hydrodynamic diameter) (table 1). In  
511 particular, the engineered platelet NPs were the most agglomerated (platelet shape could  
512 probably promote more agglomeration than the other shapes) and commercial P25 and food  
513 grade were more agglomerated than the other engineered NPs (bipyramids and rods). The  
514 variation in cellular uptake could be due to agglomeration tendency because NPs that form  
515 large agglomerates, differently from NPs that form smaller ones, precipitate at the bottom of  
516 the cell culture wells, increasing the real amount of NPs to which cells are exposed  
517 (Magdolenova et al. 2012b). Cells exposed to more NPs could probably internalize more NPs.  
518 Then, in the present study, the agglomeration tendency does not seem to have prevented the  
519 uptake of NPs in the cells, in accordance with the study of Ahlinder et al. (2013).

520 The major uptake of P25, food grade and platelet NPs could be related with higher genotoxic  
521 effect considering that, after penetration into the cells, NPs may have direct access to DNA via  
522 transport into the nucleus and/or during mitosis when the dissolution of nuclear membrane  
523 occurs. NPs interacting directly with DNA could cause DNA breakage (Magdolenova et al.,  
524 2014). Moreover, NPs, after penetration into the cells, can enhance the permeability of the  
525 lysosomal membrane, inducing the release of DNases and so causing genotoxic effects  
526 (Karlsson et al., 2010). Finally, accumulation of NPs within cells can cause aggregates of NPs  
527 that deform nucleus inducing DNA damage (Di Virgilio et al., 2010).

528 In order to quantify effects due to the photocatalytic activity of TiO<sub>2</sub>, the highest doses (80,  
529 120, 160 µg/ml) of NPs that showed a genotoxic effect were tested also in darkness (24h).  
530 Results obtained in this study showed that light exposure induced additional indirect  
531 genotoxicity, demonstrating a higher oxidative potential of TiO<sub>2</sub>-NPs after exposure with light.  
532 The presence of light increased DNA oxidative damage probably due to the photocatalytic  
533 activity of TiO<sub>2</sub>-NPs, which caused an increase of NPs ability to produce radicals. In particular,  
534 based on previous studies, the anatase crystal structure of TiO<sub>2</sub> (the same used in the present  
535 study) seems to be the most catalytic/photocatalytic crystalline structure of TiO<sub>2</sub> and seems to  
536 be activated under both ultraviolet and visible light (Warheit and Donner 2015). A recent study  
537 (De Matteis et al. 2016) demonstrated that, in particular using anatase, light is a dominant factor  
538 to induce oxidative stress and toxic effects. Also Gerloff et al. (2009) showed the increase of  
539 oxidative genotoxic effects induced by TiO<sub>2</sub>-NPs (80%/20% anatase-rutile) in the presence of  
540 interior light.

541 However, an oxidative damage (although low) was observed in the present study also in  
542 darkness as reported in the study of Gurr et al. (2005) that demonstrated that in darkness TiO<sub>2</sub>-  
543 NPs can induce oxidative DNA damage. On the contrary, Karlsson et al. (2008) and Gerloff et

544 al. (2009) found that TiO<sub>2</sub>-NPs (mixture of rutile and anatase) in darkness did not show  
545 oxidative DNA damage using the Fpg-modified Comet assay.

546 Moreover, the results obtained in this study highlight that only food grade and platelet NPs  
547 induced direct genotoxicity. However, while for food grade NPs the direct genotoxic effect  
548 remains the same both after exposure with light and in the darkness, for the commercial food  
549 grade NPs, the direct damage was higher in presence of light than in darkness. This result agree  
550 with the study of Gopalan et al. (2009); they suggest that TiO<sub>2</sub> (anatase 40 – 70 nm range) is  
551 capable of inducing higher direct genotoxic effects after simultaneous irradiation with UV,  
552 respect to genotoxicity induced in darkness. The increase of direct DNA damage after exposure  
553 with light attested by Gopalan et al. (2009) and detected for food grade NPs, remain to be  
554 explained. A possible mechanism that may lead to this effect could be related to the potential  
555 interaction of TiO<sub>2</sub>-NPs with proteins involved in DNA repair, as demonstrated by Jugan et al.  
556 (2011). Genotoxicity is not only linked to the level of DNA damage but also to the type of  
557 lesions generated and their capacity to be repaired. NPs exposure in presence of light could  
558 influence activity of proteins such as repair enzymes, resulting in DNA damage not repaired or  
559 misrepaired (Magdolenova et al. 2014). Then, the exposure with light may have caused  
560 inactivation of repair enzymes, inducing a higher direct genotoxic effect induced by food grade  
561 NPs after exposure with light respect to exposure in darkness.

562 In conclusion, the results of this study showed that the cytotoxicity was overall low (WST-1  
563 assay) and was influenced by the NP shape as well as by light exposure. According to the low  
564 cytotoxic effect, no LDH release was detected using the LDH assay.

565 Instead, genotoxicity seemed to be influenced by the cellular-uptake and the aggregation  
566 tendency of TiO<sub>2</sub>-NPs. These two aspects are probably related to different physico-chemical  
567 characteristics of NPs, such as the shape. Moreover, the presence of light enhanced the  
568 genotoxic effect of some NPs primarily increasing the oxidative stress.



569 Although more studies have to be performed in order to assess the potential toxicity of  
570 engineered NPs, the results of this preliminary study showed that engineered NPs did not  
571 induced a high cytotoxic/genotoxic effect compared to the other commercial TiO<sub>2</sub>-NPs, so they  
572 could be used for future technological applications. The results of this study are important  
573 considering that engineered NPs, due to their peculiar characteristics, could support and  
574 improve TiO<sub>2</sub>-NPs applications in different areas such as energy (i.e. use of engineered TiO<sub>2</sub>-  
575 NPs in dye-sensitized solar cells), environment (i.e. application of engineered TiO<sub>2</sub>-NPs as  
576 photocatalyst for the abatement of air and water pollutants) and health (i.e. use of engineered  
577 TiO<sub>2</sub> –NPs for the production of nanostructured coatings of orthopedic and dental prostheses  
578 exhibiting optimized interfacial properties).

579

#### 580 **Funding**

581 This work was supported by the SETNanoMetro Seventh Framework Programme project  
582 (project number 604577; call identifier FP7-NMP-2013\_LARGE-7).

583

#### 584 **Competing interests**

585 The authors declare that they have no competing interests.

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912 **Table 1**

<b>Sample</b>	<b>Particle size (nm)</b>	<b>D<sub>h</sub> DMSO (nm)</b>	<b>D<sub>h</sub> RPMI Base (nm)</b>	<b>D<sub>h</sub> RPMI Complete (nm)</b>	<b>Crystalline Phase</b>
P25	20 ± 5 quasi-spherical	107 ± 31	722 ± 246	121 ± 37	Anatase:Rutile (5:1)
Food grade	150 ± 50 undefined shape	184 ± 61	278 ± 54	184 ± 55	Anatase
Bipyramids	50 ± 9* (aspect ratio 3:2)	66 ± 20	259 ± 46	88 ± 24	Anatase
Rods	108 ± 47* (aspect ratio 1:5)	36 ± 12	1500 ± 471	39 ± 17	Anatase
Platelets	75 ± 25* (aspect ratio 8:1)	233 ± 70	281 ± 83	250 ± 82	Anatase

913

914 Table 1. Physico-chemical properties of the TiO<sub>2</sub>-NPs samples. Data are presented as mean ±  
915 standard deviation of 500 NPs for the particle size and 5 measurements for the hydrodynamic  
916 diameter (D<sub>h</sub>) of each sample. \*The particle size was calculated along the major axis of the  
917 NPs.

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926 **Figure captions**

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

931 Figure 2. Cytotoxicity measured with WST-1 (a,b) and LDH release (c,d) of BEAS-2B cells  
932 exposed to different concentrations (5–80  $\mu\text{g/ml}$ ) of commercial and engineered NPs. Control  
933 level is at 100%. Data represent effects detected after exposure with laboratory light (a,c) and  
934 in darkness (b,d). Data represent the mean % of the different wells, bars represent standard  
935 deviation.  $\ast = p < 0.05$   $\S = p < 0.001$ ; vs control cells (C-) according to ANOVA test, followed by  
936 Dunnett's test.

937 Figure 3. Effect of BEAS-2B cells exposure to commercial and engineered NPs. AC (-Fpg) =  
938 alkaline Comet assay (direct DNA damage); MC (+Fpg) = Fpg-modified Comet assay (direct  
939 + indirect DNA damage). Ox = oxidative DNA damage (tail intensity (%) in enzyme-treated  
940 cells - tail intensity (%) in enzyme-untreated cells). Exposure with laboratory light (a-e): (a)  
941 P25, (b) food grade, (c) bipyramids, (d) rods, (e) platelet NPs; exposure in darkness (f-h): (f)  
942 P25, (g) food grade, (h) platelet NPs. Data represent the mean % of tail intensity; bars represent  
943 standard deviation of two independent experiments for each experimental condition.  $\ast = p < 0.05$   
944  $\S = p < 0.001$  DNA damage vs control cells (C-). a=  $p < 0.05$  b=  $p < 0.001$  oxidative DNA damage  
945 vs control cells (C-). According to ANOVA test, followed by Dunnett's test.

946 Figure 4. 3D confocal micro-Raman imaging of BEAS-2B cells after exposure to commercial  
947 and engineered NPs. Top views (optical and 3D Raman) and 3D Raman sections are shown  
948 from the left to the right: (a) P25, (b) food grade, (c) platelet NPs, (d) bipyramids, (e) rods. 3D  
949 chemical images are built by superimposing the different maps of each cell at their  
950 corresponding focal planes and they are presented using a color meshwork i.e. blue for cell



951 tissues (methylene blue  $\nu(\text{C-C})$  ring at  $1600\text{ cm}^{-1}$ ) and red for  $\text{TiO}_2$  agglomerates (Eg band at  
952  $144\text{ cm}^{-1}$  of the anatase  $\text{TiO}_2$ ).