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

Article Type: Full Length Article

Keywords: shape-engineered TiO₂ nanoparticles; genotoxic and oxidative damage; Comet assay; cytotoxicity; Raman spectroscopy

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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.

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

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Highlights

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

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Highlights

- Cytotoxicity/genotoxicity evaluation of engineered TiO₂-NPs with different shapes
- TiO₂-NPs cytotoxicity was low, influenced by the shape and by light exposure
- Genotoxicity was influenced by cellular-uptake and aggregation tendency of TiO₂-NPs
- The presence of light enhanced the oxidative DNA damage
- It seems that shape engineered TiO₂-NPs are safer than the commercial ones

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

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List of Abbreviations

BEAS-2B – Human bronchial epithelial cells

D_h – Hydrodynamic diameter

Fpg – formamidopyrimidine glycosylase

NP – Nanoparticle

TiO_2 – Titanium dioxide

Abstract

The aim of this study was to evaluate cytotoxicity (WST-1 and LDH assays) and genotoxicity (Comet assay) of three engineered TiO₂-NPs with different shapes (bipyramids, rods, platelets) in comparison with two commercial TiO₂-NPs (P25, food grade).

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2 **genotoxicity in bronchial epithelial cells**

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28	D _h – Hydrodynamic diameter
29	Fpg – formamidopyrimidine glycosylase
30	NP – Nanoparticle
31	TiO ₂ – Titanium dioxide
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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/TS 27687:2008). Actually, many consumer products incorporates NPs. The
79 technological, medical and economic benefits of NPs are considerable, but the presence of
80 nanoparticles in the environment could cause adverse effects to humans. NPs have a grater
81 surface area per unit mass, so they potentially have an increased biological activity compared
82 to fine particles. Moreover, NPs size is comparable to size of cellular structures, so NPs
83 might potentially emulate biological molecules or interfere physically with biological
84 processes (Magdolenova *et al.* 2012a).

85 TiO₂ 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.
87 In contrast, rutile and anatase are largely used in commercial products (Jovanovic 2015).
88 TiO₂ is one of the most frequently applied NPs and it is in the top five NPs used in consumer
89 products (Shi *et al.* 2013). TiO₂-NPs produced are used primarily as a pigment owing to their
90 brightness, resistance to discoloration and high refractive index. As a pigment TiO₂-NPs are
91 incorporated in paints, plastic materials, paper, foods, medical products and cosmetics. Due
92 to its catalytic and photocatalytic properties, TiO₂ is also used as an antimicrobial agent and a
93 catalyst for purification of air and water (Bonetta *et al.* 2013, Tomankova *et al.* 2015).

94 TiO₂-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 TiO₂-NPs with
96 various shapes (e.g. rods, dots and belts) have been prepared for different applications
97 (Bernard and Curtiss 2005, Sha *et al.* 2015, Wang *et al.* 2004). In particular engineered fiber-
98 shaped nanomaterials (i.e. nanowires, nanotubes) are very attractive because showed higher
99 activity and advantages in photocatalysis, charge transfer and sensing applications due to

100 their structure (Hamilton *et al.* 2009). However, these new and enhanced properties may also
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3 101 induce higher toxicological effects upon exposure with biological tissues.

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5 102 Humans can be exposed to TiO₂-NPs via three portals of entry: oral (mainly via food
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7 103 consumption), dermal (often through cosmetic and sunscreen applications) and inhalation
8
9 104 (mainly under occupational and manufacturing conditions) (Warheit and Donner 2015).

10
11 105 Based on the evidence that TiO₂ can induce lung cancer in rats, TiO₂-NPs were classified as
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13 106 possibly carcinogenic to humans (group 2B) by the International Agency for Research on
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15 107 Cancer (IARC; Kuempel and Ruder 2012). Indeed, the inhalation and instillation of rutile and
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17 108 anatase TiO₂-NPs induced lung tumors (Xu *et al.* 2010), broncho-alveolar adenomas and
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19 109 cystic keratinizing squamous cell carcinomas (De Matteis *et al.* 2016; Mohra *et al.* 2006).
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21 110 TiO₂-NPs were also classified as potential occupational carcinogens by the National Institute
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23 111 for Occupational Safety and Health (NIOSH 2011; Chen *et al.* 2014).
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29 112 Many *in vitro* studies showed cytotoxicity, genotoxicity and oxidative effects induced by
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31 113 TiO₂-NPs through oxidants generation, inflammation and apoptosis (Jugan *et al.* 2011,
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33 114 Karlsson *et al.* 2015, Park *et al.* 2008, Shi *et al.* 2010). The potential of NPs to cause DNA
34
35 115 damage is an important aspect that needs attention because it could induce mutations and
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37 116 carcinogenesis. Physico-chemical characteristics of NPs have an important role on toxicity.
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39 117 Different studies showed that biological effects can be influenced by crystalline structure,
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41 118 size, shape, exterior area, agglomeration/aggregation and surface properties (Bhattacharya *et*
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43 119 *al.* 2009, Johnston *et al.* 2009). Some studies revealed that crystalline structure probably
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45 120 influences the induced toxicity, in particular the anatase seems to be more reactive (Sayes *et*
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47 121 *al.* 2006) and induces more toxic, genotoxic and inflammatory effects, than the rutile (Falck
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49 122 *et al.* 2009, Petkovic *et al.* 2011, Xue *et al.* 2010). However, other studies gave contradictory
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51 123 results with rutile forms being more toxic than anatase (Gurr *et al.* 2005, Numano *et al.* 2014,
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53 124 Uboldi *et al.* 2016). The effect of agglomeration/aggregation of NPs on toxicity is not well
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125 understood yet. In recent studies, some authors demonstrated that agglomeration can
126 influence NPs genotoxicity (Magdolenova *et al.* 2012b, Prasad *et al.* 2013).

127 Although physico-chemical properties of NPs can have an important role in the impact on
128 their toxicity, only few studies on shape dependent TiO₂ toxicity has been conducted (Allegri
129 *et al.* 2016, Hamilton *et al.* 2009, Park *et al.* 2013). Additional studies are needed to evaluate
130 the role of shape on TiO₂-NPs toxicity in order to produce useful data for assessing the safety
131 of engineered NPs. To address this issue, the aim of this study was to investigate cytotoxicity
132 (WST-1 and LDH) and genotoxicity (Comet assay) of three types of engineered TiO₂-NPs of
133 different shapes (bipyramids, rods and platelet NPs) in BEAS-2B (cells isolated from human
134 bronchial epithelium) in comparison with two commercial types of TiO₂-NPs (P25 and food
135 grade). Since the exposure to TiO₂-NPs mainly occurs through respiratory tract, human cells
136 of the respiratory system (such as BEAS-2B), were selected as a good cell model for *in vitro*
137 toxicology tests. All the TiO₂-NPs in this study were first physico-chemically characterized,
138 even in different culture media to study their agglomeration state, and then they were
139 biologically evaluated. In order to take into account the photocatalytic properties of the TiO₂-
140 NPs, we investigated the effects on cytotoxicity and genotoxicity on BEAS-2B under light
141 exposure and in darkness. Moreover, a modern application of Raman spectroscopy, the 3D
142 confocal Raman imaging, was used to study the uptake of the NPs within the BEAS-2B cells,
143 as the Raman spectra provide information about both organic molecules and solid NPs
144 simultaneously (Ahlinder *et al.* 2013).

146 **2. Materials and methods**

147 **2.1 Synthesis and Preparation of TiO₂ NPs dispersion**

148 Rods and bipyramids TiO₂-NPs were synthesized by the forced hydrolysis of an aqueous
149 solution of TiIV(triethanolamine)₂titanatrane (Ti(TEOAH)₂), using triethanolamine (TEOA)

150 as shape controller; pH of synthesis was adjusted by adding 1 M NaOH solution; details of
151 these procedures were previously reported (Iannarelli *et al.* 2016, Lavric *et al.* 2017). The
152 synthesis of platelet NPs was performed with a solvothermal method (Han *et al.* 2009, Zhang
153 *et al.* 2012). In a typical synthesis: a precise volume of Ti(OBu)₄ was added in a 150 ml
154 Teflon pot and the desired volume of concentrated hydrofluoric acid was added dropwise
155 under stirring. The Teflon pot was sealed and kept under stirring at high temperature (250°C)
156 for 24h in autoclave. The resulting paste was centrifuged three times and washed with
157 acetone and with water (Milli-Q) to remove the residual organics. The synthesis dispersions
158 were subjected to dialysis process (against ultrapure water, using Spectra/Por dialysis
159 membrane tubing MWCO 8–14 kDa) in order to clean the medium. To avoid agglomeration
160 and precipitation, dimethylsulfoxide (DMSO 1% in water) was added to the NPs dispersions
161 and the dispersions were homogenized using an ultra-sonication procedure, as previously
162 described by Iannarelli *et al.* (2016).
163 The same ultra-sonication procedure was employed in the preparation of the dispersion of
164 commercial TiO₂ powders, which were the P25 NPs (Evonik), extensively used in toxicity
165 studies (Karlsson *et al.* 2015, Magdolenova *et al.* 2014, Valant *et al.* 2012), and the food
166 grade NPs (Faravelli Group), incorporated in many edible products (Weir *et al.* 2012).
167 Powders of commercial TiO₂-NPs were first dispersed in a solution of DMSO (1% in water)
168 and then ultrasonicated as described above.
169 Stock solutions of both commercial and engineered TiO₂-NPs were prepared at the final
170 concentration of 2.5 mg/ml.

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

172 The dimensional characterization (size and shape) of TiO₂-NPs was carried out with SEM
173 using 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
177 applied 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 (η) 0.890 cP
183 and refractive index of water 1.3325. In order to simulate the culture medium conditions,
184 DLS analyses were conducted on dilution of TiO₂ dispersions (1:4) in a 1% DMSO aqueous
185 solution, as reference analysis, and in base and complete RPMI 1640 medium.

186 ***2.4 Raman spectroscopy analysis***

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

193 ***2.5 Cell culture and exposure***

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

197 Just before the exposure the fresh stock solution of NPs (2.5 mg/ml) was vortexed and
198 sonicated (30 min) in order to disperse the NPs. NPs (5 – 160 µg/ml) were added to the cells
199 and mixed on a shaker (10 min). The cells were exposed for 1h under laboratory light

200 (36W/840 Lumilux Cool White-36 W, 3350 lm, 4000 K-supplied from OSRAM lighting AG)

201 and then incubated at 37 °C in darkness (23h) (exposure with light). To quantify effects due

202 to the photocatalytic activity of TiO₂, cells were exposed for 24h in darkness (exposure in

203 darkness).

204 After exposure, cytotoxicity and genotoxicity assays were performed.

205 **2.6 Cytotoxicity**

206 Cell viability was assessed using Cell Proliferation Reagent WST-1 (Roche). The assay was

207 performed as previously described by Gea et al. (2018). Briefly, BEAS-2B were seeded in

208 24-well plates (5×10^4 cells/well) and exposed to NPs (5, 10, 20, 50 and 80 µg/ml, equivalent

209 to 1.3, 2.6, 5.2, 13.0, 20.7 µg/cm²). After exposure, WST-1 was added (50 µl/well) and

210 incubated for 3h (37 °C). After incubation, well contents were centrifuged and the

211 supernatants were transferred in 96-well plate to remove the interference owing to the NPs.

212 The absorbance was measured at 440 nm (Tecan Infinite Reader M200 Pro). Absorbance of

213 unexposed cells was used as negative control. Data were expressed as a percentage of

214 viability. All experiments were performed in quadruplicate (four wells for each experimental

215 condition).

216 As indicator of cell membrane damage, Lactate dehydrogenase activity was measured in cell-

217 free culture supernatants using the LDH assay kit (Cytotoxicity Detection Kit PLUS, Roche)

218 modified for NPs exposure. Briefly, BEAS-2B cells were seeded in 24-well plates (5×10^4

219 cells/well) and exposed to NPs (5, 10, 20, 50 and 80 µg/ml, equivalent to 1.3, 2.6, 5.2, 13.0,

220 20.7 µg/cm²). After exposure, the contents of each well were centrifuged to remove the

221 interference owing to the NPs. Each supernatant (100 µl) was transferred into 96-well plate,

222 mixed with Reaction Mixture (100 µl/well) and incubated for 30 min at 15 – 25 °C. After

223 incubation, Stop Solution (50 µl/well) was added and the absorbance was measured at 490

224 nm (Tecan Infinite Reader M200 Pro). Absorbance measurement of unexposed cells was

225 used as negative control, while absorbance measurement of unexposed lysed cells was used
226 as positive control. LDH release was expressed as a percentage of control cells. All
227 experiments were performed in triplicate (three wells for each experimental condition).

228 **2.7 Genotoxicity**

229 For DNA damage evaluation the BEAS-2B cells were cultured overnight in 6-well plates ($3 \times$
230 10^5 cells/well) before exposure to NPs. Cells were exposed to different doses of NPs: 20, 50,
231 80, 120 and 160 $\mu\text{g/ml}$, equivalent to 5.2, 13.0, 20.8, 31.2, 41.6 $\mu\text{g/cm}^2$. Unexposed cells and
232 cells treated with NPs dispersion liquid (DMSO 1%) were used as negative controls. After
233 exposure, cell viability was determined using trypan blue staining. The Comet assay was
234 performed according to Tice *et al.* (2000) after slight modifications (Bonetta *et al.* 2009). The
235 percentage of tail intensity was used to estimate DNA damage. A hundred randomly selected
236 cells per treatment (2 spot) were analyzed using the Comet Assay IV software (Perceptive
237 Instruments Ltd). Two independent experiments were performed for each experimental
238 condition.

239 The oxidative DNA damage was evaluated using the formamidopyrimidine glycosylase
240 (Fpg)-modified Comet assay as reported in Bonetta *et al.* (2018).

241 For each experimental point, the mean % tail intensity from enzyme untreated cells (direct
242 DNA damage) and mean % tail intensity for Fpg-enzyme treated cells (direct and indirect
243 DNA damage) were calculated. Two independent experiments were performed for each
244 experimental condition.

245 **2.8 3D confocal micro-Raman imaging spectroscopy**

246 Raman grade Calcium fluoride (CaF_2) windows (Crystran Technology srl) were employed as
247 alternative substrate instead of standard plastic substrates for cells growing due to the low
248 toxicity and almost absent background signals (Kann *et al.* 2015). The BEAS-2B cells were
249 cultured overnight in 6-well plates on a CaF_2 substrate (3×10^5 cells/well) before exposure to

250 NPs. Cells were treated with NPs (80 $\mu\text{g}/\text{ml}$, 24h). After exposure, cells were washed twice
251 with PBS and fixed with 3 ml of methanol. CaF_2 substrates were dried and stained with
252 Giemsa dye (4% Giemsa's azur eosin methylene blue solution, 4% Sorensen buffer 0.067 M
253 pH 6.8, 8 min at room temperature), then washed twice with distilled water and dried.
254 Giemsa staining is one of the standard procedures in histology, useful to evidence
255 morphological cells features, such as cell nuclei, which appear in various shades of
256 red/purple, and the cytoplasm, which appears blue.

257 3D confocal micro-Raman imaging spectroscopy of BEAS-2B cells was conducted with a
258 DXRTMxi Raman Imaging Microscope (Thermo Scientific) using a laser wavelength at 532
259 nm, a 1 mW laser power, a 100X microscope objective and a motorized stage with a 1 μm of
260 step size and a 1 μm offset. Spectra were collected in the 50–3500 cm^{-1} spectral region with a
261 grating resolution of 5 cm^{-1} , an exposure time of 0.025 s and 5 scans in total. 3D Raman
262 images were reconstructed taking the Raman peaks at 1600 cm^{-1} of methylene blue and the E_g
263 band at 144 cm^{-1} of the TiO_2 -NPs, respectively. Each cell was investigated at different focal
264 planes and a chemical image was obtained by the combination of the $\nu(\text{C-C})$ ring at 1600 cm^{-1}
265 of the methylene blue and the E_g band at 144 cm^{-1} of the TiO_2 -NPs. Since methylene blue
266 is contained in the Giemsa stain and it is widely distributed into the fixed cells, its signals
267 were considered representative of the entire volume of the cells. As far as the tracking of the
268 NPs are concerned, the E_g band at 143 cm^{-1} is the most intense signal in the molecular
269 fingerprint of the anatase TiO_2 and the region between 50 cm^{-1} and 400 cm^{-1} in the Raman
270 spectrum is usually free of the vibrational bands of biological species. Therefore, this signal
271 was selected to sensitively locate the TiO_2 -NPs inside the cells. Image J software was used in
272 the development of the 3D chemical images both for cells and TiO_2 -NPs, which were
273 superimposed using a Solidworks[®] 2016 Cad based software. 3D Raman chemical images
274 are presented using a color meshwork i.e. blue for cell tissues and red for TiO_2 agglomerates.

275

276 **2.9 Statistical analysis**

277 IBM SPSS software (ver. 24.0) was used to perform statistical analysis. The results of WST-
278 1, LDH and Comet assays are presented as the mean±standard deviation. Differences
279 between exposed and control cells were tested by ANOVA followed by Dunnett's test
280 procedure. Differences between light and dark exposure were tested by ANOVA, followed by
281 the Tukey's test procedure. Data were considered statistically different for a p-value less than
282 0.05.

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284 **3. Results**

285 **3.1 Raman characterization of NPs and size distribution**

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

293 These NPs were also characterized by Dynamic Light Scattering (DLS) as a quick method for
294 sizing and determining the state of NP agglomeration. For each kind of sample, the
295 agglomeration in 1% DMSO aqueous solution, in base RPMI and complete RPMI (Fig. 1f-j)
296 were compared. In all the TiO₂ materials considered for this study, the agglomeration state
297 increase in base RPMI, while the size distribution in DMSO and in complete RPMI is quite
298 similar.

299 The crystalline composition of the TiO₂-NPs, analyzed by Raman spectroscopy, showed a
300 typical fingerprint of the anatase TiO₂ (Fig. S.1) with the characteristic phonon bands E_g at
301 143 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
302 investigated NPs. Since P25 is a known mixture of anatase and rutile (5:1), with also a small
303 amount of amorphous TiO₂ (Ohtani *et al.* 2010), its Raman spectrum still retains all the
304 typical anatase Raman bands but it also contains two small shoulders at 450 cm⁻¹ and 600 cm⁻¹,
305 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 TiO₂-NPs under study such as shape,
306 particle size, hydrodynamic diameter in different liquid media and the crystalline phase are
307 summarized in Table 1.

309 **3.2 Cytotoxicity**

310 The results of the effects of different TiO₂-NPs concentration on cell viability (WST-1 assay)
311 are reported in Fig. 2a (exposure with light) and in Fig. 2b (exposure in darkness).

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

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

321 Considering the exposure in darkness, a lower cytotoxic effect was observed for commercial
322 P25 NPs with respect to light exposure because a slight decrease in viability was observed for
323 P25 NPs only at the highest dose (80 µg/ml) (p<0.05). As reported after exposure with light,

324 no cytotoxic effect was observed for the other commercial NPs (food grade). About
325 engineered NPs, the exposure in darkness did not modify the cytotoxic effect of bipyrramids
326 NPs resulting in a viability reduction starting from the dose of 50 $\mu\text{g/ml}$ ($p<0.001$) as
327 reported in the experiment with light. In contrast, in the darkness, rods NPs showed a lower
328 cytotoxic effect than observed with light because a slight decrease in viability was observed
329 for rod NPs only starting from the dose of 20 $\mu\text{g/ml}$ ($p<0.05$ or $p<0.001$). As during the
330 exposure with light, platelet NPs induced a decrease in viability; the cytotoxic effect was
331 significant starting from a less dose (10 $\mu\text{g/ml}$, $p<0.05$) than in the experiment with light (50
332 $\mu\text{g/ml}$).

333 The results of the effects of different TiO_2 -NPs concentration on lactate dehydrogenase
334 activity (LDH assay) has been reported in Fig. 2c (exposure with light) and in Fig. 2d
335 (exposure in darkness).

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

338 **3.3 Genotoxicity**

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

341 Considering the exposure with laboratory light, no genotoxic effect was showed in enzyme
342 untreated cells (direct DNA damage) for commercial P25 NPs (Fig. 3a). On the other hand, a
343 dose-dependent increase of DNA damage was observed for these NPs in enzyme treated cells
344 (direct and indirect DNA damage) respect to the control cells ($p<0.05$ or $p<0.001$), with the
345 exception of the last dose (160 $\mu\text{g/ml}$) that induced a DNA damage equal to 80 $\mu\text{g/ml}$. A
346 significant oxidative damage was observed for P25 NPs starting from 50 $\mu\text{g/ml}$ ($p<0.05$ or
347 $p<0.001$). The results obtained with the other commercial NPs (food grade)(Fig. 3b) showed
348 the presence of a significant dose-response DNA damage both in enzyme untreated cells and

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349 in enzyme treated cells starting from 50 µg/ml. Moreover, the difference between the two
350 effects resulted significant starting from 20 µg/ml ($p<0.05$ or $p<0.001$) highlighting an
351 oxidative damage induced by food grade NPs.

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

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

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

370 Considering the exposure in darkness, no genotoxic effect was observed for commercial P25
371 NPs in enzyme untreated cells (direct DNA damage) (Fig. 3f) as reported in the experiment
372 with light (Fig. 3a). However, in the enzyme treated cells a dose-response DNA damage
373 (direct and indirect DNA damage) was observed with respect to control cells ($p<0.05$ or

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374 $p < 0.001$), but oxidative DNA damage was lower than in the experiment with light ($p < 0.05$ or
375 $p < 0.001$). The commercial food grade NPs induced a significant dose-response DNA damage
376 both in enzyme untreated cells and in enzyme treated cells ($p < 0.001$ and $p < 0.05$ respectively)
377 (Fig. 3g). However, the DNA damage resulted in both cases lower than in the experiment
378 with light $p < 0.05$ or $p < 0.001$) and an oxidative damage was induced only at the highest dose
379 (160 $\mu\text{g/ml}$) ($p < 0.05$).

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

386 **3.4 Confocal micro-Raman spectroscopy**

387 Confocal micro-Raman imaging spectroscopy was used to evaluate the uptake and the
388 distribution of the different types of TiO_2 -NPs within the cells. 3D chemical images are built
389 by superimposing the different maps of each cell at their corresponding focal planes and they
390 are presented using a color meshwork i.e. blue for cell tissues and red for TiO_2 agglomerates.
391 At least five cells were analyzed to provide statistically significant results. As the sections of
392 Fig. 4 show, the uptake of the TiO_2 -NPs by the cells was mainly demonstrated for P25, food
393 grade and platelet NPs (Fig. 4a,b,c) while no TiO_2 signal was registered inside the cells for
394 bypyramids and rods (Fig. 4d,e).

396 **4. Discussion**

397 The aim of this study was to investigate the cytotoxicity and genotoxicity of three different
398 shapes of TiO_2 -NPs and to compare them with two commercially available TiO_2 -NPs. The

399 factors taken into account for this study were: i) the physico-chemical properties of the
400 particles such as shape, particle size, agglomeration state in culture media, crystalline phase,
401 ii) the ability of the particles to induce cytotoxicity and genotoxicity, iii) the increase of the
402 toxicological effects under light exposure due to the photocatalytic activity of TiO₂ and iv)
403 the uptake of the NPs by human cells.

404 Published results on toxicity of TiO₂-NPs show high variability. Reasons for this variability
405 include physico-chemical characteristics of NPs, different methods for prepare NPs
406 dispersions, differences in NPs size and dispersion stability, and different exposure protocols
407 (Charles *et al.*, 2018). The characteristics of NPs dispersion can be influenced by medium
408 components, such as serum proteins, and by NPs properties (size, shape, surface charge,
409 surface coating etc.) (Huk *et al.* 2015). According to the study of Prasad *et al.* (2013), the
410 present results showed that in all the TiO₂-NPs dispersions, the agglomeration state increases
411 in base RPMI (without serum), while the size distribution in DMSO and in complete RPMI
412 medium (with serum) is quite similar. The different agglomeration state is probably due to
413 the ability of metal oxide NPs to adsorb proteins onto their surface, forming a “protein
414 corona” which favors less agglomeration in complete medium, which contains more proteins
415 (Prasad *et al.* 2013). Considering the results obtained, complete medium was selected as
416 cytotoxicity/genotoxicity assay medium.

417 The viability of BEAS-2B treated with commercial and engineered TiO₂-NPs after exposure
418 with light or in darkness was assessed using the WST-1 assay.

419 Commercial TiO₂-NPs induced low or no viability reduction (P25 and food grade,
420 respectively) detected by WST-1 assay, such that these results are in agreement with some
421 reports on the cytotoxicity of commercial TiO₂-NPs on BEAS-2B cells (Bhattacharya *et al.*
422 2009, Falck *et al.* 2009). Other studies showed that commercial TiO₂-NPs induced
423 cytotoxicity on BEAS-2B (Shi *et al.* 2010, Ursini *et al.* 2014). According to the results on

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424 P25, in the study of Prasad *et al.* (2013) 100 µg/ml of commercial P25 NPs produced a
425 viability decrease in BEAS-2B cells after 24h exposure. In contrast, Park *et al.* (2008) found
426 that exposure of BEAS-2B cells to commercial P25 (5-40 µg/ml) for 24h led to significant
427 cell death, both in a time- and concentration-dependent manner. Instead, fewer studies have
428 been performed using commercial food grade TiO₂-NPs. Proquin *et al.* (2017) tested these
429 NPs on different cell lines: on Caco-2, they observed cytotoxicity, while, according to our
430 results, on HCT116 they did not observe any cytotoxic effect up to the concentration of 100
431 µg/cm², confirming that the cytotoxicity can be influenced by the use of different cell lines.
432 Recently, the scientific community have produced reference NPs, which have been well
433 characterized. About evaluation of these reference TiO₂-NPs, Di Bucchianico *et al.* (2016)
434 assessed cytotoxic effects of some of these NPs (anatase 50-150 nm, anatase 5-8 nm, rutile
435 20-28 nm) in BEAS-2B cells; according to the present results the study shows in general no
436 or low effects at the doses tested (2-100 µg/ml).
437 The data demonstrated that cytotoxicity was slightly affected by light exposure, which
438 induced an increase of cellular damage after incubation with commercial P25 and engineered
439 rods.
440 Comparing the results of WST-1 assay and LDH release, the first showed low cytotoxic
441 effect at the doses tested, while the second did not show any cytotoxicity in both exposure
442 protocols. The discrepancy between LDH release and WST-1 data suggests that the viability
443 reduction may be caused by apoptosis, a cell death pathway in which the plasma membrane is
444 maintained, as observed in other studies (Schilirò *et al.* 2015). This is in accordance with
445 previous studies, which demonstrated that TiO₂-NPs could cause apoptosis in BEAS-2B cells
446 (Park *et al.* 2008, Shi *et al.* 2010).

1 447 Results of Comet assay in presence of light and in darkness showed a significant DNA
2 448 damage induced by commercial P25 and food grade NPs and engineered platelet NPs, while
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4 449 no genotoxicity was observed with other NPs.
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7 450 Considering that the uptake of NPs could involve interactions of NPs with DNA, the
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9 451 observed genotoxic effect could be related to the presence of P25, food grade and platelet
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11 452 NPs into the BEAS-2B detected in the present study and by other studies (Bhattacharya *et al.*
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13 453 2009, Park *et al.* 2008). The higher uptake of P25, food grade and platelet NPs could be
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15 454 explained with the higher agglomeration tendency (higher hydrodynamic diameter) (table 1).
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17 455 Indeed, as supposed by Magdolenova *et al.* (2012b), TiO₂-NPs that form large agglomerates,
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19 456 differently from NPs that form smaller ones, precipitate at the bottom of the cell culture
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21 457 wells, increasing the real amount of NPs to which the cells are exposed. After penetration
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23 458 into the cells, these NPs may have direct access to DNA via transport into the nucleus and/or
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25 459 during mitosis when the dissolution of nuclear membrane occurs, so they could cause DNA
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27 460 breakage (Magdolenova *et al.* 2014). Other hypothesized mechanisms that can induce the
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29 461 observed DNA damage are that NPs (or metal ions released from particles) can enhance the
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31 462 permeability of the lysosomal membrane, inducing the release of DNases (Karlsson *et al.*
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33 463 2010) or that NPs aggregates can deform nucleus causing DNA damage (Di Virgilio *et al.*
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35 464 2010).
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39 465 In addition, to quantify effects due to the photocatalytic activity of TiO₂, the highest doses
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41 466 (80, 120, 160 µg/ml) of NPs that showed a genotoxic effect were tested also in darkness
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43 467 (24h). Results showed that light exposure induced additional indirect genotoxicity,
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45 468 demonstrating a higher oxidative potential of TiO₂-NPs after exposure with light than in
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47 469 darkness. The presence of light increased DNA oxidative damage probably due to the
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49 470 photocatalytic activity of TiO₂-NPs, which caused an increase of NPs ability to produce
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51 471 radicals. In particular, based on previous studies, the anatase crystal structure of TiO₂ (used in
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472 this study) seems to be the most catalytic/photocatalytic crystalline structure of TiO₂ and
473 seems to be activated under both ultraviolet and visible light (Warheit and Donner 2015). A
474 recent study (De Matteis *et al.* 2016) demonstrated that, in particular using anatase, light is a
475 dominant factor to induce oxidative stress, TiO₂-NPs degradation and toxic effects.
476 According to the present results, Gerloff *et al.* (2009) showed direct and oxidative genotoxic
477 effects induced by TiO₂-NPs (80%/20% anatase-rutile) only in the presence of interior light.
478 Karlsson *et al.* (2008) found that TiO₂-NPs (mixture of rutile and anatase) in darkness did not
479 show oxidative DNA damage using the Fpg-Comet assay (Karlsson *et al.* 2010). However, as
480 observed in the present study, also in darkness TiO₂-NPs can induce oxidative DNA damage,
481 although lower than damage induced by light exposure. This result was observed also in the
482 study of Gurr *et al.* (2005).
483 The results obtained highlight that food grade and engineered platelet NPs induced direct
484 genotoxicity also in darkness. For the commercial food grade NPs the damage was lower than
485 in presence of light. This result agree with the study of Gopalan *et al.* (2009); they suggest
486 that TiO₂ (anatase 40 – 70 nm range) is capable of inducing higher direct genotoxic effects
487 after simultaneous irradiation with UV, respect to genotoxicity induced in darkness. The
488 increase of direct DNA damage after exposure with light, attested by Gopalan *et al.* (2009)
489 and detected for food grade NPs, remain to be explained. A possible mechanisms, that may
490 lead to this effect, could be related to the potential interaction of TiO₂-NPs with proteins
491 involved in DNA repair, as demonstrated by Jugan *et al.* (2011). Genotoxicity is not only
492 linked to the level of DNA damage but also to the type of lesions generated and their capacity
493 to be repaired. NPs exposure in presence of light could influence activity of proteins such as
494 repair enzymes, resulting in DNA damage not repaired or misrepaired (Magdolenova *et al.*
495 2014).

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496 In conclusion, cytotoxicity of NPs was low, and was influenced by the NP shape as well as
497 by light exposure. Instead, genotoxicity seemed to be influenced by the cellular-uptake and
498 the aggregation tendency of TiO₂-NPs. These two aspects are probably related to different
499 physico-chemical characteristics of NPs, such as the shape. Moreover, the presence of light
500 enhanced the genotoxic effect of some NPs primarily increasing the oxidative stress. In
501 summary, the results obtained indicate that the TiO₂-NPs shape may play a critical role in the
502 potential genotoxicity and light can influence cytotoxicity and genotoxicity of both
503 commercial and engineered TiO₂-NPs. The results obtained suggests that these shape
504 engineered TiO₂-NPs are probably safer than the commercial ones.

505

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509

510 **Competing interests**

511 The authors declare that they have no competing interests.

512

513 **References**

514 Ahlinder, L., Ekstrand – Hammarstrom, B., Geladi, P., and Osterlund, L., 2013. Large uptake
515 of titania and iron oxide nanoparticle in the nucleus of lung epithelial cells as measured by
516 raman imaging and multivariate classification. *Biophysical Journal*, 105, 310 – 319.

517 Allegri, M., Bianchi, M.G., Chiu, M., Varet, G., Costa, A.L., Ortelli, S., Blosi, M., Bussolati,
518 O., Poland, C.A. and Bergamaschi, E., 2016. Shape-related toxicity of titanium dioxide
519 nanofibres. *PLoS ONE*, 11(3), 1 – 21.

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520 Bernard, A.S. and Curtiss, L.A., 2005. Prediction of TiO₂ nanoparticle phase and shape
521 transitions controlled by surface chemistry. *Nano Letters*, 5: 1261 – 1266.

522 Bhattacharya, K., Davoren, M., Boertz, J., Schins, R.P., Hoffmann, E. and Dopp, E., 2009.
523 Titanium dioxide nanoparticles induce oxidative stress and DNA-adduct formation but not
524 DNA-breakage in human lung cells. *Particle and Fibre Toxicology*, 6, 17 – 27.

525 Bonetta, S., Bonetta, S., Motta, F., Strini, A., Carraro, E., 2013. Photocatalytic bacterial
526 inactivation by TiO₂-coated surfaces. *AMB Express*, 3: 59 – 66.

527 Bonetta, S., Bonetta, S., Schilirò, T., Ceretti, E., Feretti, D., Covolo, L., Vannini, S., Villarini,
528 M., Moretti, M., Verani, M., Carducci, A., Bagordo, F., De Donno, A., Bonizzoni, S.,
529 Bonetti, A., Pignata, C., Carraro, E., Gelatti, U., MAPEC_LIFE Study Group, Gilli, G.,
530 Romanazzi, V., Gea, M., Festa, A., Viola, G.C.V., Zani, C., Zerbini, I., Donato, F., Monarca,
531 S., Fatigoni, C., Levorato, S., Salvatori, T., Donzelli, G., Palomba, G., Casini, B., De Giorgi,
532 M., Devoti, G., Grassi, T., Idolo, A., Panico, A., Serio, F., Furia, C., Colombi, P., 2018.
533 Mutagenic and genotoxic effects induced by PM_{0.5} of different Italian towns in human cells
534 and bacteria: The MAPEC_LIFE study. *Environmental Pollution*, doi:
535 <https://doi.org/10.1016/j.envpol.2018.11.017>.

536 Bonetta, S., Giannotti, V., Bonetta, S., Gosetti, F., Oddone, M. and Carraro, E., 2009. DNA
537 damage in A549 cells exposed to different extracts of PM_{2.5} from industrial, urban and
538 highway sites. *Chemosphere*, 77, 1030 – 1034.

539 Charles, S., Jomini, S., Fessard, V., Bigorgne-Vizade, E., Rousselle, C., Michel, C., 2018.
540 Assessment of the *in vitro* genotoxicity of TiO₂ nanoparticles in a regulatory context.
541 *Nanotoxicology*, 12(4): 357 – 374.

542 Chen, T., Yan, J. and Li, Y., 2014. Genotoxicity of titanium dioxide nanoparticles. *Journal of*
543 *Food and Drug Analysis*, 22, 95 – 104.

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544 Collins, A., El Yamani, N., Dusinska, M., 2017. Sensitive detection of DNA oxidation
545 damage induced by nanomaterials. *Free Radical Biology and Medicine*, 107, 69 – 76.

546 De Matteis, V., Cascione, M., Brunetti, V., Toma, C.C. and Rinaldi, R., 2016. Toxicity
547 assessment of anatase and rutile titanium dioxide nanoparticles: the role of degradation in
548 different pH conditions and light exposure. *Toxicology in Vitro*, 37, 201 – 210.

549 Di Bucchianico, S., Cappellini, F., Le Bihanic, F., Zhang, Y., Dreij, K. and Karlsson, H.L.,
550 2016. Genotoxicity of TiO₂ nanoparticle assessed by mini-gel Comet assay and micronucleus
551 scoring with flow cytometry. *Mutagenesis*, 0, 1 – 11.

552 Di Virgilio, A.L., Reigosa, M., Arnal, P.M. and Fernández Lorenzo de Mele, M., 2010.
553 Comparative study of the cytotoxic and genotoxic effects of titanium oxide and aluminium
554 oxide nanoparticles in Chinese hamster ovary (CHO-K1) cells. *Journal of Hazardous*
555 *Materials*, 177, 711 – 718.

556 Falck, G.C.M., Lindberg, H.K., Suhonen, S., Vippola, M., Vanhala, E., Catalan, J.,
557 Savolainen, K. and Norppa, H., 2009. Genotoxic effects of nanosized and fine TiO₂. *Human*
558 *& Experimental Toxicology*, 28(6 – 7), 339 – 352.

559 Gerloff, K., Albrecht, C., Boots, A.W., Förster, I. and Schins, R.P.F., 2009. Cytotoxicity and
560 oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells. *Nanotoxicology*,
561 3(4), 355–364.

562 Gopalan, R.C., Osman, I.F., Amani, A., De Matas, M. and Anderson, D., 2009. The effect of
563 zinc oxide and titanium dioxide nanoparticles in the Comet assay with UVA photo activation
564 of human sperm and lymphocytes. *Nanotoxicology*, 3(1), 33 – 39.

565 Gurr, J.R., Wang, A.S., Chen, C.H. and Jan, K.Y., 2005. Ultrafine titanium dioxide particles
566 in the absence of photoactivation can induce oxidative damage to human bronchial epithelial
567 cells. *Toxicology*, 213, 66 – 73.

1
2
3
4
5 568 Hamilton, R.F., Wu, N., Porter, D., Buford, M., Wolfarth, M. and Holian, A., 2009. Particle
6
7 569 length-dependent titanium dioxide nanomaterials toxicity and bioactivity. *Particle and Fibre*
8
9 570 *Toxicology*, 6, 35 – 46.
10
11 571 Han, X., Kuang, Q., Jin, M., Xie, Z. and Zheng, L., 2009. Synthesis of Titania Nanosheets
12 572 with a high Percentage of Exposed (001) Facets and Related Photocatalytic Properties.
13 573 *Journal of the American Chemical Society*, 131 (9), 3152 – 3153.
14
15 574 Huk, A., Collins, A.R., El Yamani, N., Porredon, C., Azqueta, A., de Lapuente, J. and
16
17 575 Dusinska, M., 2015. Critical factors to be considered when testing nanomaterials for
18
19 576 genotoxicity with the comet assay. *Mutagenesis*, 30,85 – 88.
20
21 577 Iannarelli, L., Giovannozzi, A.M., Morelli, F., Viscotti, F., Bigini, P., Maurino, V., Spoto, G.,
22
23 578 Martra, G., Ortel, E., Hodoroaba, V., Rossi, A.M. and Diomedede, L., 2016. Shape engineered
24
25 579 TiO₂ nanoparticles in *Caenorhabditis elegans*: a Raman imaging based approach to assist
26
27 580 tissue-specific toxicological studies. *RSC Adv*, 6, 70501 – 70509.
28
29 581 ISO/TS 27687:2008. Nanotechnologies- Terminology and definitions for nano-objects-
30
31 582 Nanoparticle, nanofibre and nanoplate.
32
33 583 Johnston, H.J., Hutchison, G.R., Christensen, F.M., Peters, S., Hankin, S. and Stone, V.,
34
35 584 2009. Identification of the mechanisms that drive the toxicity of TiO₂ particulates: the
36
37 585 contribution of physicochemical characteristics. *Particle and Fibre Toxicology*, 6, 33.
38
39 586 Jovanovic, B., 2015. Critical review of public health regulations of titanium dioxide, a human
40
41 587 food additive. *Integrated Environmental Assessment and Management*, 11, 10 – 20.
42
43 588 Jugan, M.L., Barillet, S., Simon-Deckers, A., Herlin-Boime, N., Sauvaigo, S., Douki, T. and
44
45 589 Carriere, M., 2011. Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA
46
47 590 repair activity in A549 cells. *Nanotoxicology*, 6(5), 501 – 513.
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

591 Kann, B., Offerhaus, H.L., Windbergs, M. and Otto, C., 2015. Raman microscopy for cellular
1
2 592 investigations – from single cell imaging to drug carrier uptake visualization. *Advanced Drug*
3
4 593 *Delivery Reviews*, 89, 71 – 90.
5
6
7 594 Karlsson, H.L., Cronholm, P., Gustafsson, J. and Möller, L., 2008. Copper oxide
8
9 595 nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon
10
11 596 nanotubes. *Chemical Research in Toxicology*, 21, 1726 – 1732.
12
13
14 597 Karlsson, H.L., Di Bucchianico, S., Collins, A. and Dusinska, M., 2015. Can the Comet
15
16 598 Assay be used reliably to detect nanoparticle-induced genotoxicity? *Environmental and*
17
18 599 *Molecular Mutagenesis*, 56, 82 – 96.
19
20
21 600 Karlsson, H.L., 2010. The Comet assay in nanotoxicology research. *Analytical and*
22
23 601 *Bioanalytical Chemistry*, 398, 651-666.
24
25
26 602 Kuempel, E.D. and Ruder, A., 2012. Titanium dioxide (TiO₂). IARC Monograph 93.
27
28
29 603 Lavric, V., Isopescu, R., Maurino, V., Pellegrino, F., Pellutiè, L., Ortel, E. and Hodoroaba,
30
31 604 A., 2017. New Model for Nano-TiO₂ Crystal Birth and Growth in Hydrothermal Treatment
32
33 605 Using an Oriented Attachment Approach. *Crystal Growth & Design*, 17 (11), 5640–5651.
34
35
36 606 Magdolenova, Z., Bilanicova, D., Pojana, G., Fjellsbo, L.M., Hudecova, A., Hasplova, K.,
37
38 607 Marcomini, A. and Dusinska, M., 2012b. Impact of agglomeration and different dispersion of
39
40 608 titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity.
41
42 609 *Environmental Monitoring and Assessment*, 14, 455 – 464.
43
44
45 610 Magdolenova, Z., Collins, A., Kumar, A., Dhawan, A., Stone, V. and Dusinska, M., 2014.
46
47 611 Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered
48
49 612 nanoparticles. *Nanotoxicology*, 8(3), 233 – 278.
50
51
52 613 Magdolenova, Z., Lorenzo, Y., Collins, A. and Dusinska, M., 2012a. Can standard
53
54 614 genotoxicity tests be applied to nanoparticles? *Journal of Toxicology and Environmental*
55
56 615 *Health, Part A*, 75, 13 – 15.
57
58
59
60
61
62
63
64
65

616 Mohra, U., Ernst, H., Roller, M. and Pott, F., 2006. Pulmonary tumor types induced in Wistar
1 rats of the so-called 19-dust study. *Experimental and Toxicologic Pathology*, 58(1), 13 – 20.
2
3
4
5 618 NIOSH. National Institute for Occupational Safety and Health: Occupational Exposure to
6
7 619 Titanium Dioxide. In Current Intelligence Bulletin 63. Cincinnati: National Institute for
8
9
10 620 Occupational Safety and Health; 2011.
11
12 621 Numano, T., Xu J., Futakuchi, M., Fukamachi, K., Alexander, D. B., Furukawa, F., Kanno,
13
14
15 622 J., Hirose, A. Tsuda, H., Suzuim, M., 2014. Comparative study of toxic effects of anatase and
16
17 623 rutile type nanosized titanium dioxide particles *in vivo* and *in vitro*. *Asian Pacific Journal of*
18
19 624 *Cancer Prevention*, 15 (2), 929 – 935.
20
21
22 625 Ohtani, B., Prieto-Mahaney, O.O., Li, D. and Abe, R., 2010. What is Degussa (Evonik) P25?
23
24 626 Crystalline composition analysis, reconstruction from isolated pure particles and
25
26
27 627 photocatalytic activity test. *Journal of Photochemistry and Photobiology A: Chemistry*, 216,
28
29 628 179 – 182.
30
31
32 629 Park, E.J., Lee, G., Shim, H., Kim, J., Cho, M. and Kim, D., 2013. Comparison of toxicity of
33
34 630 different nanorod-type TiO₂ polymorphs *in vivo* and *in vitro*. *Journal of Applied Toxicology*,
35
36 631 34, 357 – 366.
37
38
39 632 Park, E.J., Yi, J., Chung, Y.H., Ryu, D.Y., Choi, J. and Park, K., 2008. Oxidative stress and
40
41 633 apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *Toxicology*
42
43 634 *Letters*, 180(3), 222 – 229.
44
45
46 635 Petkovic, J., Zegura, B., Stevanovic, M., Drnovsek, N., Uskokovic, D., Novak, S. and Filipic,
47
48
49 636 M., 2011. DNA damage and alterations in expression of DNA damage responsive genes
50
51 637 induced by TiO₂ nanoparticles in human hepatoma HepG2 cells. *Nanotoxicology*, 5, 341 –
52
53 638 353.
54
55
56 639 Prasad, R.Y., Wallace, K., Daniel, K.M., Tennant, A.H., Zucker, R.M., Strickland, J., Dreher,
57
58 640 K., Klingerman, A.,D., Blackman, C.F. and De Marini, D.M., 2013. Effect of treatment
59
60
61
62
63
64
65

641 media on the agglomeration of titanium dioxide nanoparticles: impact on genotoxicity,
1
2
3 642 cellular interaction, and cell cycle. *ACS Nano*, 7, 1929 – 1942.
4
5 643 Proquin, H., Rodriguez-Ibarra, C., Moonen, C.G.J., Urrutia Ortega, I.M., Briede, J.J., de Kok,
6
7 644 T.M., van Loveren, H. and Chirino, Y.I., 2017. Titanium dioxide food additive (E171)
8
9 645 induces ROS formation and genotoxicity: contribution of micro and nano-sized fractions.
10
11 646 *Mutagenesis*, 32, 139 – 149.
12
13 647 Sayes, C.M., Wahi, R., Kurian, P.A., Liu, Y., West, J.L., Ausman, K.D., Warheit, D.B. and
14
15 648 Colvin, V.L., 2006. Correlating nanoscale titania structure with toxicity: a cytotoxicity and
16
17 649 inflammatory response study with human dermal fibroblast and human lung epithelial cells.
18
19 650 *Toxicological Sciences*, 92,174 – 185.
20
21
22 651 Schilirò, T., Bonetta, S., Alessandria, L., Gianotti, V., Carraro, E. and Gilli, G., 2015. PM10
23
24 652 in a background urban site: chemical characteristics and biological effects. *Environmental*
25
26 653 *Toxicology and Pharmacology*, 39(2), 833 – 844.
27
28
29 654 Sha, B., Gao, W., Cui, W., Wang, L. and Xu, F., 2015. The potential health challenges of
30
31 655 TiO₂ nanomaterials. *Journal of Applied Toxicology*, 35, 1086 – 1101.
32
33
34 656 Shi, H., Magaye, R., Castranova, V. and Zhao, J., 2013. Titanium dioxide nanoparticles: a
35
36 657 review of current toxicological data. *Particle and Fibre Toxicology*, 10, 15.
37
38
39 658 Shi, Y., Wang, F., He, J., Yadav, S. and Wang, H., 2010. Titanium dioxide nanoparticles
40
41 659 cause apoptosis in BEAS-2B cells through caspase 8/t-Bid-independent mitochondrial
42
43 660 pathway. *Toxicology Letters*, 196, 21 – 27.
44
45
46 661 Tice, R.R., Agurell, E., Anderson, D., Burlison, B., Hartmann, A., Kobayashi, H., Miyamae,
47
48 662 Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F., 2000. Single cell gel/Comet assay: guidelines for
49
50 663 in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*,
51
52 664 35, 206 – 221.
53
54
55
56
57
58
59
60
61
62
63
64
65

1 665 Tomankova, K., Horakova, J., Harvanova, M., Malina, L., Soukupova, J., Hradilova, S.,
2 666 Kejllova, K., Malohlava, J., Licman, L., Dvorakova, M., Jirova, D. and Kolarova, H., 2015.
3
4 667 Cytotoxicity, cell uptake and microscopic analysis of titanium dioxide and silver
5
6 668 nanoparticles in vitro. *Food and Chemical Toxicology*, 82, 106 – 115.
7
8
9 669 Tompsett, G.A., Bowmaker, G.A., Cooney, R.P., Metson, J.B., Rodgers, K.A. and Seakins,
10
11 670 J.M., 1995. The Raman spectrum of brookite, TiO₂ (Pbca, Z = 8). *Journal of Raman*
12
13 671 *Spectroscopy*, 26, 57 – 62.
14
15
16 672 Uboldi, C., Urban, P., Gilliland, D., Bajak, E., Valsami-Jones, E., Ponti, J., Rossi, F., 2016.
17
18 673 Role of the crystalline form of titanium dioxide nanoparticles: rutile, and not anatase, induces
19
20 674 toxic effects in Balb/3T3 mouse fibroblasts. *Toxicology in Vitro*, 31: 137 – 145.
21
22
23 675 Ursini, C.L., Cavallo, D., Fresegna, A.M., Ciervo, A., Maiello, R., Tassone, P., Buresti, G.,
24
25 676 Casciardi, S. and Iavicoli, S., 2014. Evaluation of cytotoxic, genotoxic and inflammatory
26
27 677 response in human alveolar and bronchial epithelial cells exposed to titanium dioxide
28
29 678 nanoparticles. *Journal of Applied Toxicology*, 34, 1209 – 1219.
30
31
32 679 Valant, J., Iavicoli, I. and Drobne, D., 2012. The importance of a validated standard
33
34 680 methodology to define in vitro toxicity of nano- TiO₂. *Protoplasma*, 9, 493 – 502.
35
36
37 681 Wang, W., Gu, B., Liang, L., Hamilton, W.A. and Wesolowski, D.J., 2004. Synthesis of
38
39 682 rutile (α - TiO₂) nanocrystals with controlled size and shape by low-temperature hydrolysis:
40
41 683 effects of solvent composition. *Journal of Physical Chemistry B*, 108, 14789 – 14792.
42
43
44 684 Warheit, D.B. and Donner, E.M., 2015. Risk assessment strategies for nanoscale and fine-
45
46 685 sized titanium dioxide particles: recognizing hazard and exposure issues. *Food and Chemical*
47
48 686 *Toxicology*, 85, 138 – 147.
49
50
51 687 Weir, A., Westerhoff, P., Fabricius, L. and Von Goetz, N., 2012. Titanium dioxide
52
53 688 nanoparticles in food and personal care products. *Environmental Science and Technology*,
54
55 689 46(4), 2242 – 2250.
56
57
58
59
60
61
62
63
64
65

690 Xu, J., Futakuchi, M., Iigo, M., Fukamachi, K., Alexander, D.B., Shimizu, H., Sakai, Y.,
691 Tamano, S., Furukawa, F., Uchino, T., Tokunaga, H., Nishimura, T., Hirose, A., Kanno, J.
692 and Tsuda, H., 2010. Involvement of macrophage inflammatory protein 1a (MIP1a) in
693 promotion of rat lung and mammary carcinogenic activity of nanoscale titanium dioxide
694 particles administered by intra-pulmonary spraying. *Carcinogenesis*, 31, 5927 – 5935.

695 Xue, C., Wu, J., Lan, F., Liu, W., Yang, X., Zeng, F. and Xu, H., 2010. Nano titanium
696 dioxide induces the generation of ROS and potential damage in HaCaT cells under UVA
697 irradiation. *Journal for Nanoscience and Nanotechnology*, 10, 8500 – 8507.

698 Zhang, J., Wang, J., Zhao, Z., Yu, T., Feng, J., Yuan, Y., Tang, Z., Liu, Y., Li, Z. and Zou,
699 Z., 2012. Reconstruction of the (001) surface of TiO₂ nanosheets induced by the fluorine-
700 surfactant removal process under UV-irradiation for dye-sensitized solar cells. *Physical
701 Chemistry Chemical Physics*, 14 (14), 4763–4769.

706 **Table**

Sample	Particle size (nm)	D _h DMSO (nm)	D _h RPMI Base (nm)	D _h RPMI Complete (nm)	Crystalline Phase
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	$108 \pm 47^*$	36 ± 12	1500 ± 471	39 ± 17	Anatase
	(aspect ratio 1:5)				
Platelets	$75 \pm 25^*$	233 ± 70	281 ± 83	250 ± 82	Anatase
	(aspect ratio 8:1)				

707

708 Table 1. Physico-chemical properties of the TiO₂-NPs samples. Data are presented as mean ±
709 standard deviation of 500 NPs for the particle size and 5 measurements for the hydrodynamic
710 diameter (D_h) of each sample. *The particle size was calculated along the major axis of the
711 NPs.

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720 **Figure captions**

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

725 Figure 2. Cytotoxicity (a,b) and lactate dehydrogenase (LDH) release (expressed as a
726 percentage) (c,d) of BEAS-2B cells exposed to different concentrations (5–80 µg/ml) of
727 commercial and engineered NPs. Control level is at 100%. Data represent effects detected

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728 after exposure with light (a,c) and in darkness (b,d). Bars represent the mean %, error bars
729 represent standard error of mean. * $p < 0.05$; *vs* control cells (C-) according to ANOVA,
730 followed by Dunnett's test.

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

737 Figure 4. 3D confocal micro-Raman imaging of BEAS-2B cells after exposure to commercial
738 and engineered NPs. Top views (optical and 3D Raman) and 3D Raman sections are shown
739 from the left to the right: (a) P25, (b) food grade, (c) platelet NPs, (d) bipyramids, (e) rods.
740 3D chemical images are built by superimposing the different maps of each cell at their
741 corresponding focal planes and they are presented using a color meshwork i.e. blue for cell
742 tissues (methylene blue $\nu(\text{C-C})$ ring at 1600 cm^{-1}) and red for TiO_2 agglomerates (Eg band at
743 144 cm^{-1} of the anatase TiO_2).

Figure 1

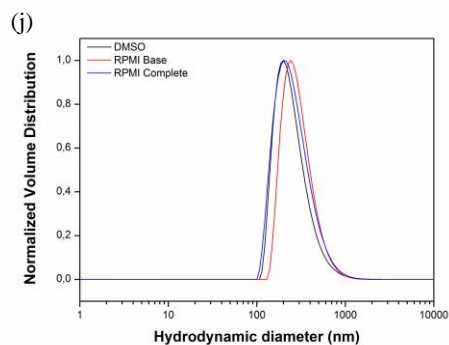
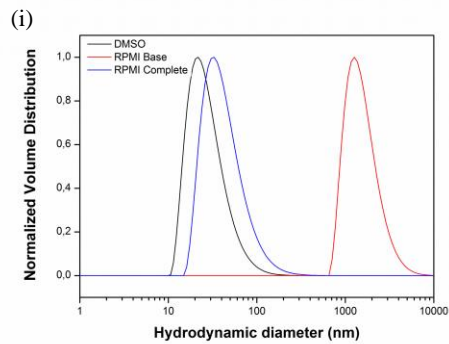
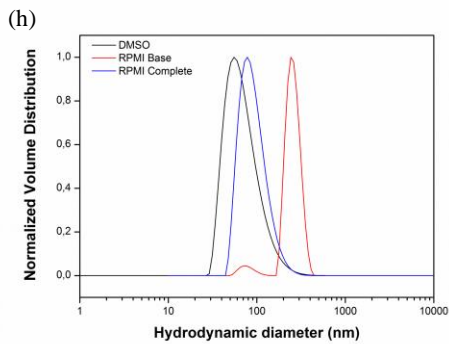
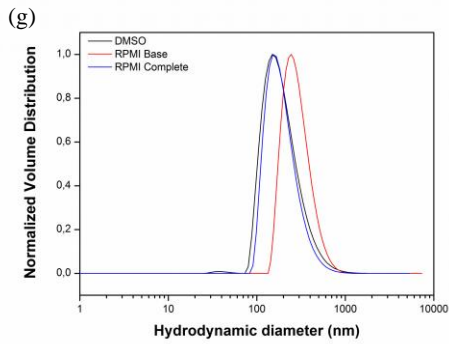
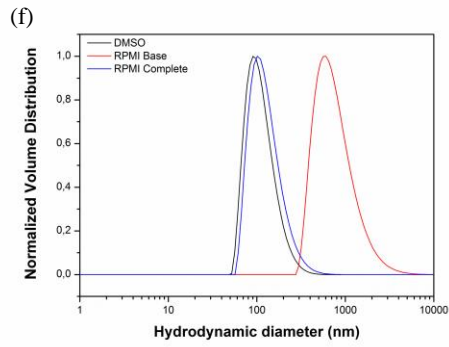
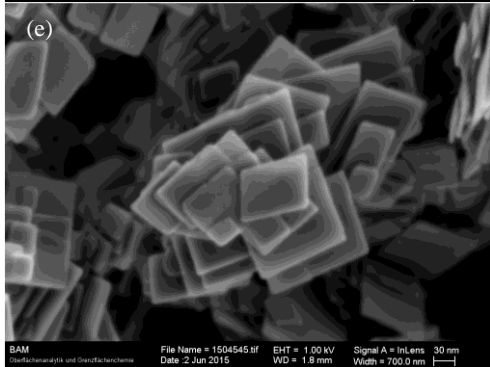
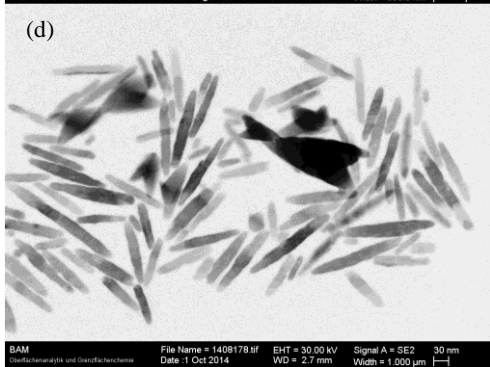
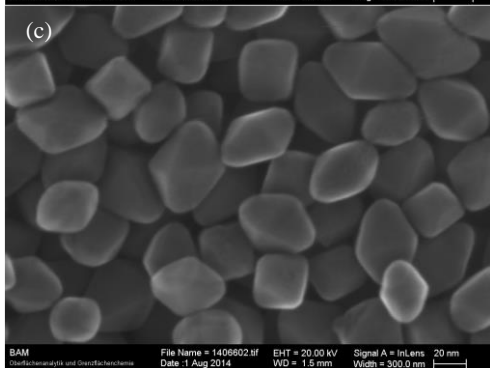
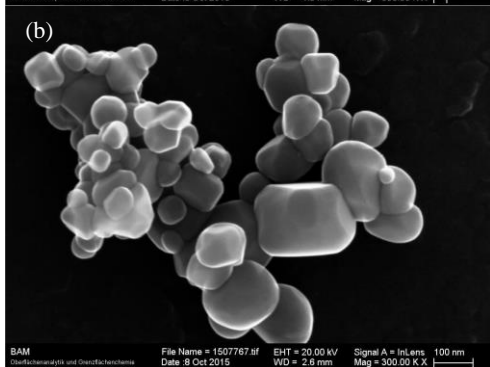
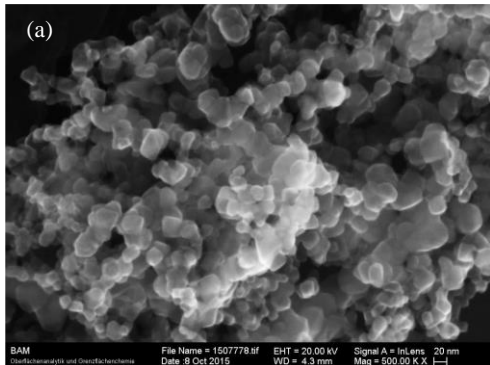
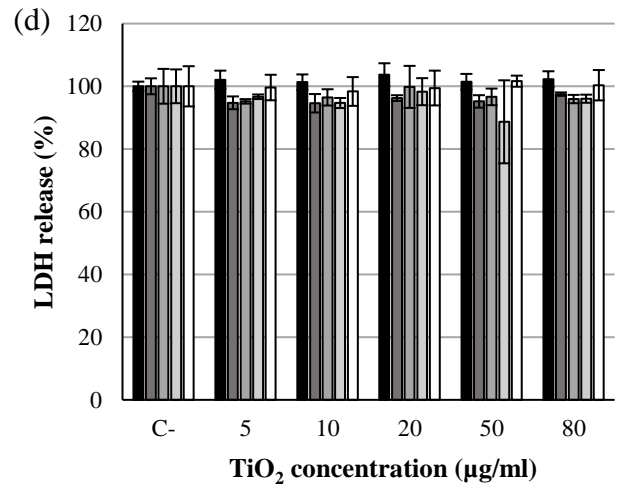
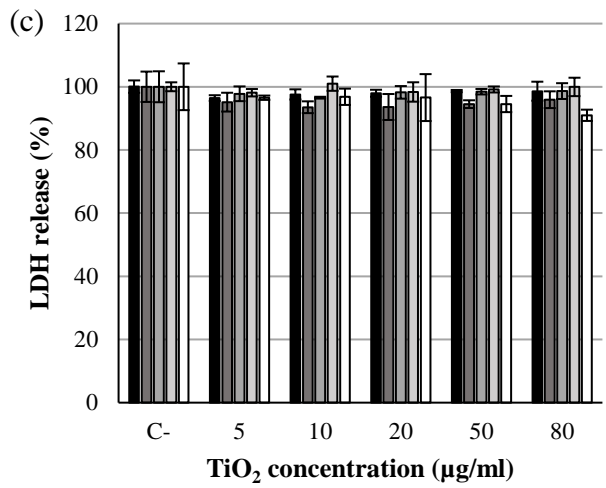
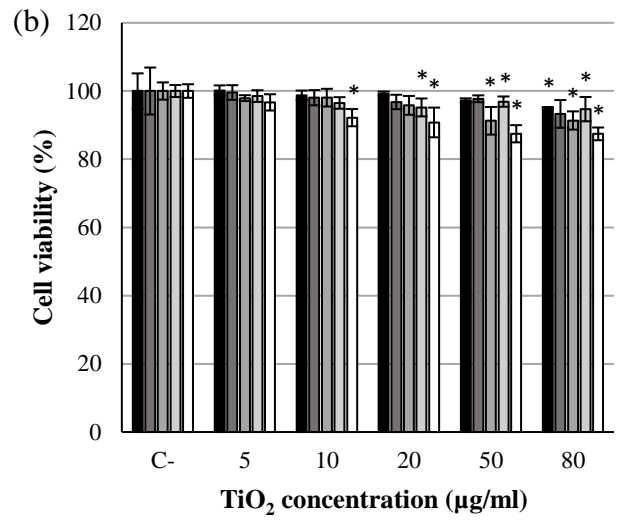
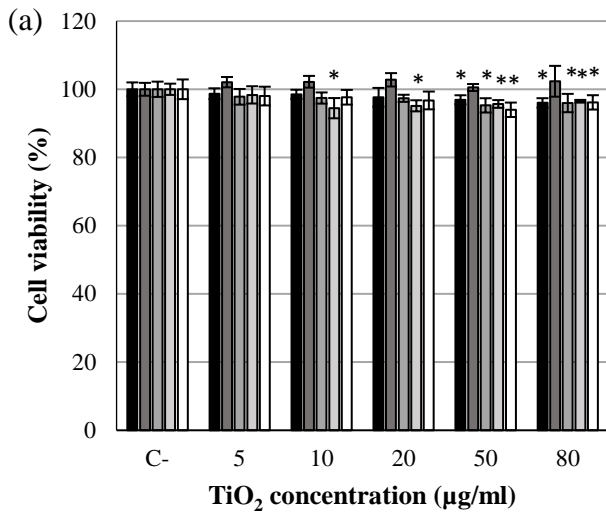


Figure 2



■ P25 ■ Food grade ■ Bipyramids □ Rods □ Platelet NPs

Figure 3

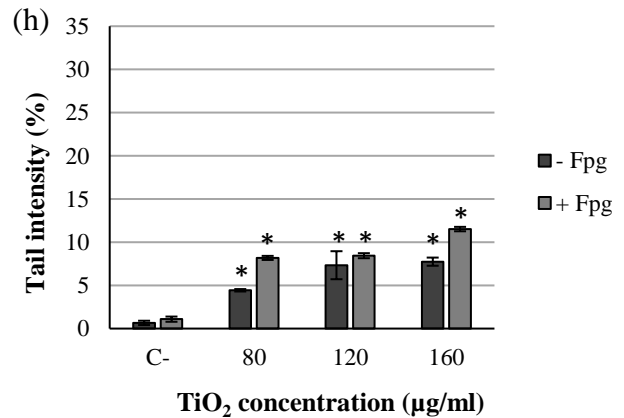
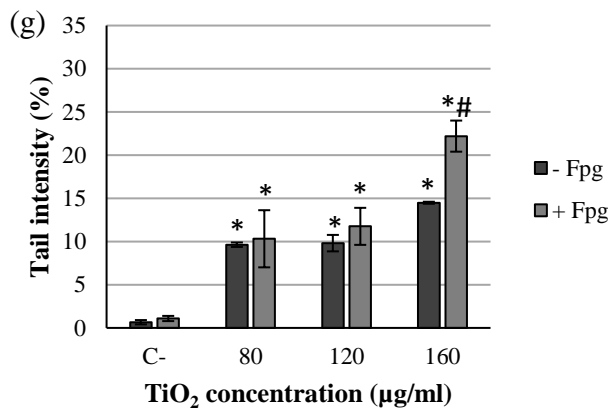
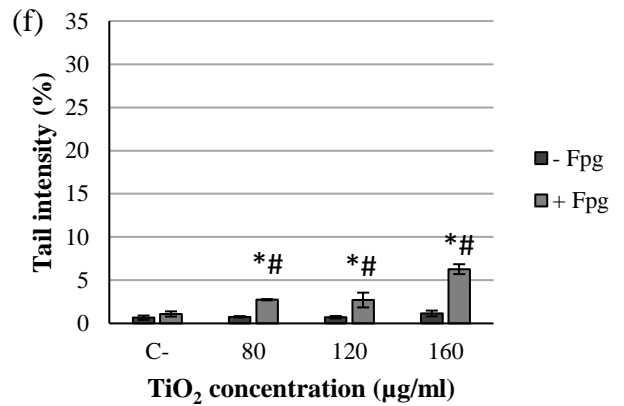
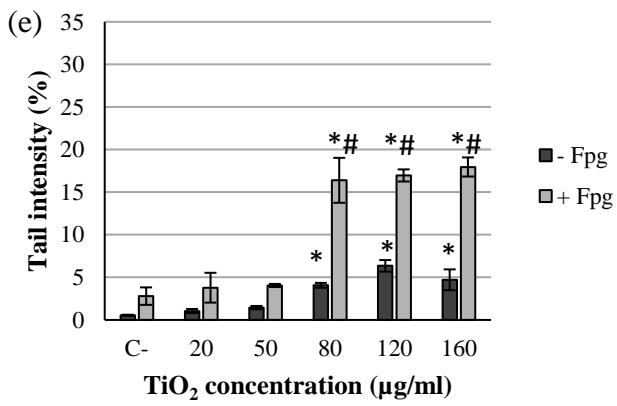
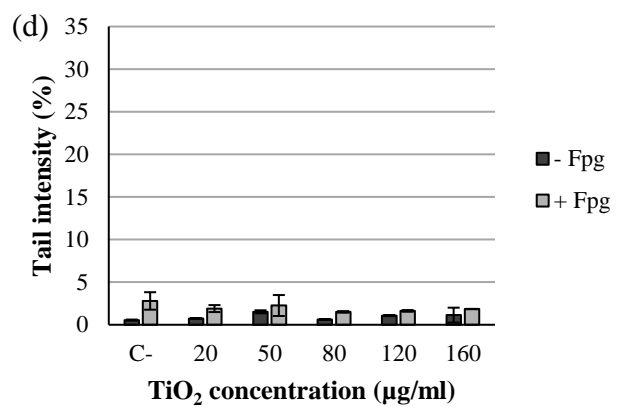
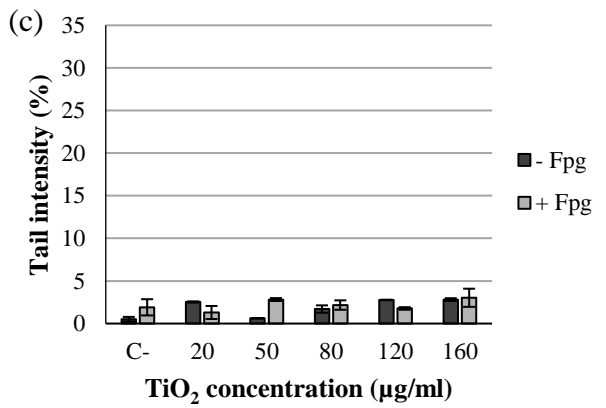
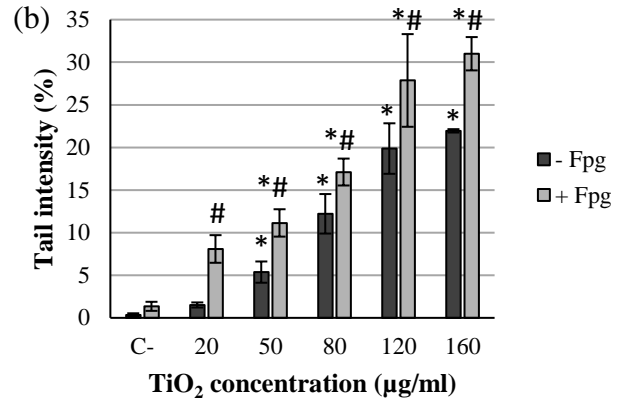
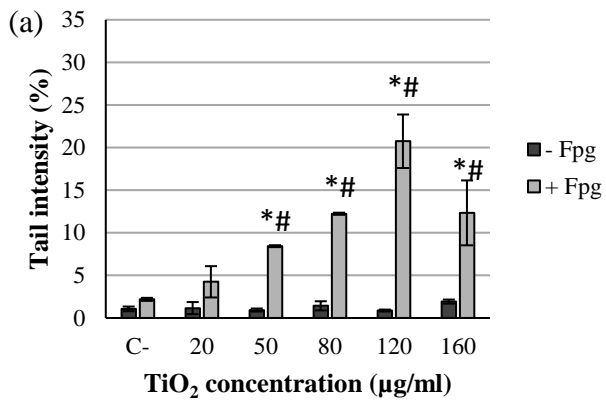
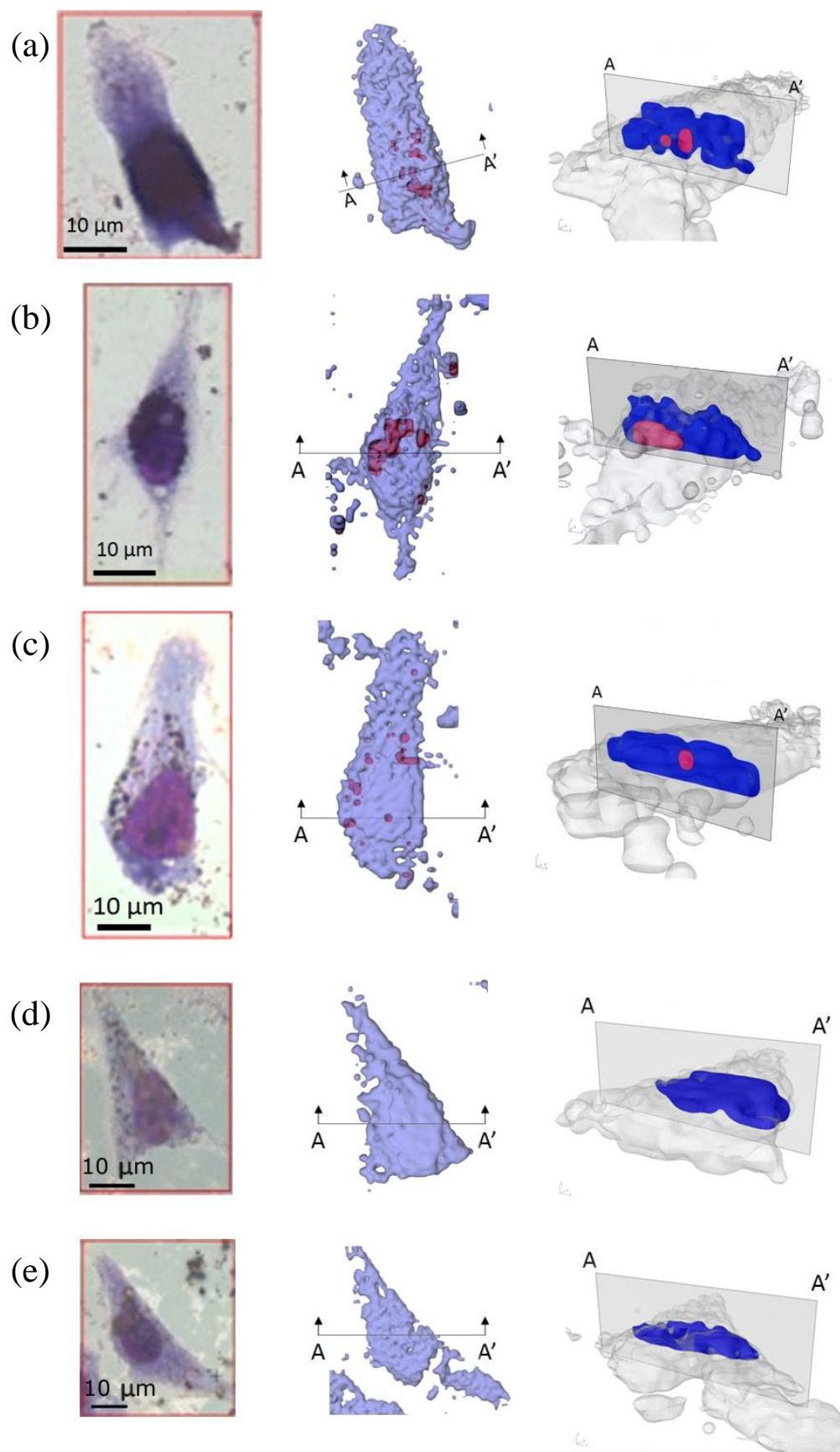


Figure 4



Supplemental

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