

Biological reactivity of TiO₂ nanoparticles assessed by ex vivo testing

Janez Valant · Damjana Drobne

Received: 16 March 2011 / Accepted: 1 June 2011 / Published online: 19 June 2011
© Springer-Verlag 2011

Abstract Isolated digestive gland epithelium from a model invertebrate organism was used in an ex vivo system to assess the potential of nanoparticulate TiO₂ to disrupt cell membranes. Primary particle size, surface area, concentration of particles in a suspension, and duration of exposure to TiO₂ particles were all found to have effects, which are observed at concentrations of nano-TiO₂ as low as 1 µg mL⁻¹. The test system employed here can be used as a fast screening tool to assess biological potential of nanoparticles with similar chemical composition but different size, concentration, or duration of exposure. We discuss the potential of ex vivo tests to avoid some of the limitations of conventional in vitro tests.

Keywords TiO₂ particles · Ex vivo testing · Nanotoxicity · Biological potential of nanoparticles

Handling Editor: Peter Nick

J. Valant (✉) · D. Drobne
Department of Biology, Biotechnical Faculty,
University of Ljubljana,
Večna pot 111,
1000 Ljubljana, Slovenia
e-mail: janez.valant@bf.uni-lj.si

D. Drobne
Centre of Excellence in Advanced Materials and Technologies
for the Future (CO NAMASTE), Jožef Stefan Institute,
Jamova 39,
1000 Ljubljana, Slovenia

D. Drobne
Centre of Excellence in Nanoscience and Nanotechnology
(CO Nanocenter), Jožef Stefan Institute,
Jamova 39,
1000 Ljubljana, Slovenia

Introduction

Because biological reactivity of nanoparticles is expected to be a function of their physicochemical properties, it has been suggested (Warheit 2008; Hussain et al. 2009) that detailed characteristics of nanoparticles should be provided together with toxicity data. It is important, however, to recognize that the properties of nanoparticles can be affected by the medium in which they are dispersed, and it is advisable to characterize the properties of nanoparticles in the test medium as well as in the solid form. Despite much original research and several review papers on nanotoxicity of engineered nanoparticles, the characteristics actually responsible for the biological reactivity have still not been identified. Singh et al. (2009) listed the results of a number of genotoxicity and cytotoxicity studies of nanomaterials and concluded that there are significant inconsistencies in the literature and that it is difficult to draw conclusions as to the physicochemical features of nanomaterials that promote effects on biological systems. They explain that while the response to nanoparticles is controlled by their large surface area, the available surface area is modified by many parameters related to the media in which the nanoparticles are suspended. Biological reactivity of nanoparticles cannot be ascribed solely to their dissolution (Poynton et al. 2011).

Assessment of the toxic potential of nanoparticles with in vitro tests is perhaps the fastest and most convenient approach (Park et al. 2009). The most important advantages of in vitro cell tests are that they represent well-established methodologies, use small set-ups, have low costs, few ethical problems, and are easily interpretable. They use large numbers of replicates and the test set-ups can be miniaturized and even automated (Hartung and Daston 2009). However, there are some limitations to conventional

in vitro tests. These are related mainly to the absence of intercellular interactions and cell defense mechanisms. In addition, cell densities in cell cultures are typically less than 1% of those in tissue and most cell systems are composed only of one cell type. It has been shown that frequently used cancer cells have many, sometimes tens of thousands of mutations (Frank and Nowak 2004; Ponten 2001) including loss of partial or complete chromosomes (Hartung and Daston 2009) and are arguably not representative of a natural tissue or organ.

In the work presented in this paper, we limited some of the constraints of in vitro tests by making use of an ex vivo test in which an isolated single-layer epithelium, e.g., the digestive gland of a model invertebrate organism, was used as a toxicological test system for the testing effect of TiO₂ particles.

The ability of TiO₂ nanoparticles to destabilize cell membrane after in vitro exposure of cell lines has already been noted by some authors (Thevenot et al. 2008; Simon-Deckers et al. 2008; Lee et al. 2009). Nanosized TiO₂ is known to disrupt cell membrane stability as a result of its photocatalytic properties (Amezaga-Madrid et al. 2003). Some authors also have explained cell membrane destabilization as a result of direct interaction between nanoparticles and the lipid bilayer (Banaszak 2009), and others explain cell membrane destabilization as resulting from lipid peroxidation, a consequence of oxidative stress (Wang et al. 2009). Destabilization of the cell membrane is undoubtedly among the primary effects of nano-TiO₂ and, therefore, suitable as a measure of the biological potential of such nanoparticles.

In the work presented here, the isolated epithelium was incubated in different suspensions of nanoparticles for a selected period of time, after which changes in cell membrane stability were assessed as a measure of the nanoparticulate reactivity. In this way, we tested the biological potential of TiO₂ particles. TiO₂ particles are commercially available with variety of physicochemical characteristics and are thus well suited for use in a comparative nanotoxicity study. In addition, since it is known that, unlike other metal oxide nanoparticles, TiO₂ particles do not produce ionic species and so any effect could not be attributed to dissolved metal ions.

The aim of this study was to test whether the response of digestive gland epithelium to TiO₂ nanoparticles is related to the primary size of the particles, their surface area, the zeta potential of a suspension, or duration of exposure or concentration. We expected that isolated digestive gland epithelium would allow identification of dose and time response to nanoparticles of similar chemical composition but different sizes. The reactivity of the nanoparticles was assessed by their potential to destabilize cell membranes. We discuss advantages of the ex vivo test system which avoids some of the limitations of conventional in vitro tests.

Materials and methods

Chemicals

Acridine orange (AO), ethidium bromide (EB), NaCl, KCl, MgCl₂, glucose, and TRIS were purchased from Merck (Darmstadt, Germany). AO, EB, and nanoparticulate TiO₂, were suspended in a physiological solution modified for use with *Porcellio scaber* (248 mM NaCl, 8 mM KCl, 5 mM MgCl₂, 5 mM glucose, and 10 mM Tris in bidistilled water). All suspensions of TiO₂ nanoparticles were prepared with this solution.

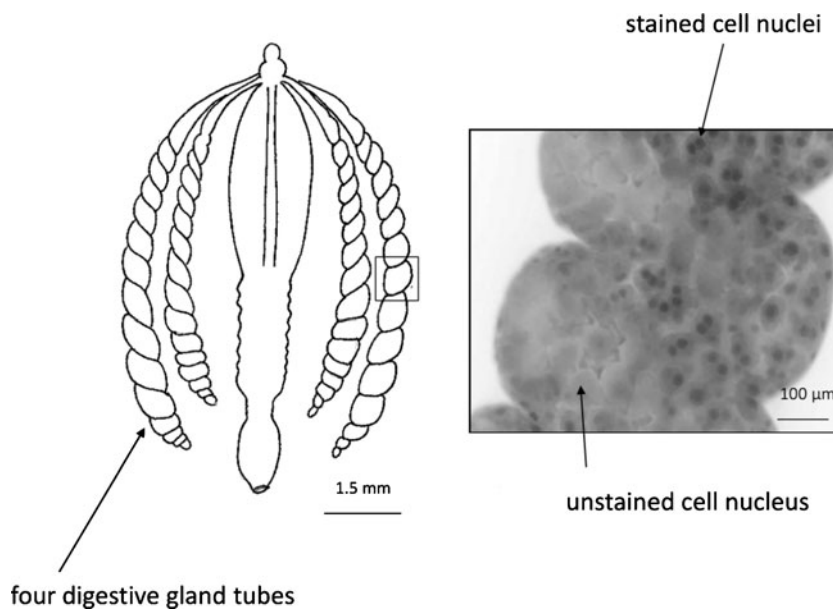
Data on crystalline structure and average size for low and medium size nanoparticles were provided by the manufacturer (Sigma-Aldrich). The following different sizes and forms of TiO₂ were tested for their potential to destabilize cell membranes: 130 nm (rutile), 30 nm (anatase with traces of rutile; Sigma-Aldrich, Steinheim, Germany), and 15 nm (anatase; Sigma-Aldrich, Steinheim, Germany). We also characterized the tested nanoparticles in this laboratory.

Model tissue

Experiments were conducted on isolated digestive glands (hepatopancreas) of a model invertebrate *Porcellio scaber* (Isopoda, Crustacea). Adult animals, body weight of 30 mg, from laboratory stock cultures were selected for the study. After decapitation, the four digestive gland tubes were gently pulled out with tweezers and placed directly into either physiological solution as a control, in a suspension of nanoparticles, or in a solution of Cu²⁺ ions. Each animal has four gland tubes, and each one of these was treated separately. Cell membrane stability was assessed by a modified acridine orange/ethidium bromide (AO/EB) method, as described previously (Valant et al. 2009) on approximately 200 cells per gland tube (Fig. 1). The authors describe the AO/EB method applied on isolated digestive gland exposed in vivo. Here, the digestive glands were isolated and incubated in a suspension of nanoparticles for a certain period of time.

The relevance of using digestive gland cells (hepatopancreas) of the model organism (*P. scaber*, Isopoda, Crustacea) in nanoparticle studies of biological effects is twofold. First, the digestive glands have intestinal, hepatic, and pancreatic functions (Zimmer 2002). They are the main site of synthesis and secretion of digestive enzymes, absorption of nutrients, storage of metabolic reserves (lipids and glycogen), and excretion of wastes. As such, hepatopancreas is a suitable model system for investigating the reactivity of ingested chemicals (Ferreira et al. 2010). Second, experiments with isolated digestive glands are highly controllable, allow optional selection of exposure duration, and produce easy interpretable data.

Fig. 1 Schematic diagram of the digestive system of a model experimental animal *Porcellio scaber* (left). A pair of digestive gland tubes is situated next to the gut tube. Light micrograph of a part of digestive gland tube (right). The AO/EB assay is based on assessing the portion of stained nuclei/unstained nuclei (see Valant et al. 2009)



Characterization of nanoparticles

Nanoparticles were examined with transmission electron microscopy (TEM), dynamic light scattering (DLS), Brunauer–Emmett–Teller (BET), and X-ray powder diffraction (XRD) techniques. The zeta potential of the suspension media was also measured.

For TEM, the dispersions of nanoparticles were applied to carbon-coated grids, dried at room temperature, and examined with a 200 keV field emission transmission electron microscope (Philips CM 100, Koninklijke Philips Electronics, at the Jožef Stefan Institute, Ljubljana).

The dispersions of nanoparticles (100 μg mL⁻¹) were inspected by DLS using a 3D DLS-SLS spectrometer (FRITSCH, Analysette 12, Dynasizer). A HeNe laser operating at a wavelength of 632.8 nm was used as the light source and scattering was measured at an angle of 90°. Suspensions were prepared on the physiologic solution and measured immediately after preparation and after 18 h.

Samples were dried and degassed with nitrogen prior to BET analysis (Tristar 3000, Micrometrics Co., Norcross, GA, USA) which provided information about the surface area of solid material.

TiO₂ samples were also monitored by XRD using a Bruker AXS D4 Endeavor diffractometer (Karlsruhe, Germany) with Cu-Kα₁ radiation and a Sol-X energy dispersive detector within the angular range of 20° < 2θ < 80°, a step size of 0.04°, and a collection time of 3 s.

Zeta potentials of TiO₂ particle suspensions (1,000 μg mL⁻¹) were measured with ZetaPals, (Brookhaven Instrument Corporation) in the physiologic solution used as the test medium.

Experimental set-up and AO/EB assay

A single digestive gland tube (hepatopancreas) was placed in an unsonicated suspension of TiO₂ nanoparticles or in a control solution for different periods of time, the time frame being selected on the basis of preliminary experiments. The exposure time was selected as the maximum time in which cell membrane of control gland epithelium cells remained unaffected by the experiential conditions. We selected the following durations of exposure: 0.5, 1, 3, 6, and 18 h.

The exposure concentrations of suspensions of TiO₂ nanoparticles, selected on the basis of preliminary experiments and literature data were: 0, 0.1, 1, 10, 100, and 1,000 μg mL⁻¹ of nano-TiO₂.

Ex vivo AO/EB assay was validated with relevant positive control. Cell membrane destabilization was induced with incubation of digestive gland tubes in a solution of Cu²⁺ ions (100 μg mL⁻¹). Copper ions stimulate production of excess levels of ROS, which lead to oxidative stress and subsequently to cell membrane destabilization (Regoli et al. 1998).

After exposure, each hepatopancreatic tube was placed in the AO/EB mixture for 5 min and subsequently on a microscope slide where it was immediately photographed by an Axioimager.Z1 fluorescent microscope (Zeiss) using two different sets of filters (Zeiss, Axioimager.Z1). An excitation filter of 450–490 nm and an emission filter of 515 nm (filter set 09) were used to visualize AO and EB stained nuclei while the excitation filter of 365 nm and the emission filter of 397 nm (filter set 01) were used to visualize nuclei stained with EB only. Cell membrane integrity was assessed by micrographic examination of a

portion of stained nuclei in each investigated region (Valant et al. 2009). In all, 200 nuclei from each gland tube were examined and a portion of the stained nuclei was assessed visually. Twelve hepatopancreatic tubes were exposed to each concentration of TiO₂ nanoparticles, thus in all, 12×6 hepatopancreatic tube glands were experimentally exposed and 18 animals were sacrificed.

Statistical analysis

Data were analyzed using standard statistical methods. The difference in the medians of measured parameter in exposed and unexposed groups was tested with the non-parametric Mann–Whitney *U* test. All calculations were done using STATGRAPHICS Plus 4.0 statistics software. Statistical differences between exposed and control animals were categorized into three groups with different numbers of stars assigned (**p*<0.05; ****p*<0.005). Hydrodynamic radii were determined using nanoQ software.

Results

Characteristics of TiO₂ nanoparticles

TEM reveals the shape and size of tested TiO₂ nanoparticles (Figs. 2a–c). All three types of TiO₂ were composed of particles of different sizes. In the smallest, the predominant size was elongated spherical, in the larger and in the bulk nanoparticles, the spherical shape prevailed. The largest particles tested were several hundred nanometers in diameter. The images of the three tested particles clearly show significant differences in the primary particle sizes, notwithstanding some overlap between groups.

DLS instrument enables the determination of hydrodynamic radii of particles in extremely turbid suspensions by a so-called 3D cross-correlation technique that successfully eliminates multiple scattering of light. In fresh suspensions, the average values of hydrodynamic radius of 10, 30, and 130 nm nanoparticles were 110, 240, and 860 nm, respectively. In suspensions which were kept in fridge for 18 h, the average values of hydrodynamic radius of 10, 30, and 130 nm were 230, 410, and 1,500 nm, respectively.

The BET method was used to assess the surface area of TiO₂ samples. The primary particle sizes of TiO₂ nanoparticles were approximately 10, 30, and 130 nm, and the surface areas were 144 m² g⁻¹, 46 m² g⁻¹ for 30 nm, and 11.7 m² g⁻¹, respectively. The size and surface area correspond with the data provided by the supplier.

XRD revealed the crystal form of the TiO₂ samples. The 10, 30, and 130 nm TiO₂ nanoparticles were in anatase, anatase with traces of rutile and rutile crystal forms, respectively.

The zeta potential values of 10 nm, 30 nm particles, and bulk TiO₂, suspended in a physiologic solution were -14, -18, and -25 mV, respectively.

Ex vivo effect of TiO₂ suspensions and Cu²⁺ ions on cell membrane stability

No significant effect on cell membrane stability was noted after 0.5, 1, 3, or 6 h incubation of hepatopancreatic gland tubes in suspensions of any of three TiO₂ particle suspensions. After incubation for 18 h, TiO₂ particles were found to destabilize cell membrane of digestive glands.

Cell membranes were significantly destabilized when digestive gland tubes were incubated in 1, 10, 100, and 1,000 μg mL⁻¹ of the 10 nm TiO₂ nanoparticles. Larger nanoparticles, with a primary particle size of 30 nm also significantly destabilized the cell membrane, but only at higher concentrations of 100 or 1,000 μg mL⁻¹. This concentration-dependent response was a threshold effect. This means that there is no effect unless the concentration is high enough. After that, also the increasing concentration did not increase the intensity of effect. An increase of even three orders of magnitude in exposure concentration failed to produce a systematic response (Figs. 3a, b). Bulk sized TiO₂ nanoparticles had no effect on cell membrane stability (Fig. 3c).

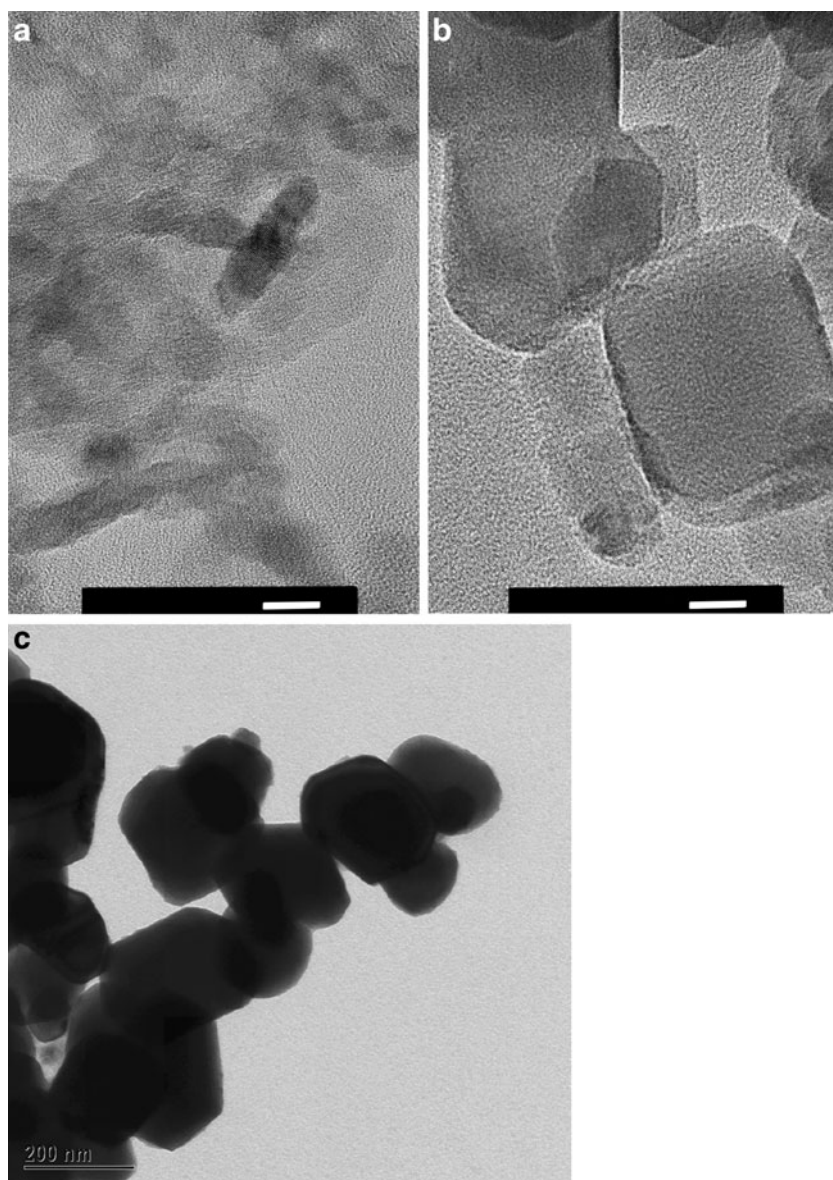
Cell membranes were also significantly destabilized when digestive gland tubes were incubated in a solution of Cu²⁺ ions (Fig. 3a–c).

Discussion

Evidence from ex vivo tests suggests that primary particle size, particle surface area, concentration of particles, particle shape, and duration of exposure to TiO₂ particles are all partly responsible for the biological potential. The ex vivo test system employed in this study provides evidence that the cell membrane of isolated digestive gland epithelium is affected at the lower concentration of TiO₂ nanoparticles (1 μg mL⁻¹). This is among the lowest concentrations of nano-TiO₂ reported to exert biological effects after 18 h of incubation. The most biologically potent materials were anatase TiO₂ nanoparticles with primary size of 10 nm, surface area of 144 m² g⁻¹, and zeta potential of -14 mV, indicating moderate suspension stability.

Currently, over 50 scientific papers on the effects of nano-TiO₂ tested in vitro have been published. Some of these (Sayes et al. 2006; Soto et al. 2007; Sayes and Warheit 2008; Simon-Deckers et al. 2008; Braydich-Stolle et al. 2009; Fujita et al. 2009; Hussain et al. 2009; Karlsson et al. 2009; Pan et al. 2009; Heinlaan et al. 2008) provide

Fig. 2 a–c Transmission electron micrographs of TiO₂ particles tested in our study. **a** TEM micrograph in 10 nm TiO₂ nanoparticles; **b** TEM micrograph in 30 nm TiO₂ nanoparticles; **c** TEM micrograph bulk TiO₂ particles. Scale bar in (a) and (b) is 10 nm



comparative data on the effects of different formulations of TiO₂ particles tested in the identical biological model and offer valuable information on the toxicity of the nanoparticles in question. It may not however, be straightforward to correlate nanotoxicity with physicochemical characteristic of particles from these reports. Namely, different authors tested different formulations, such as different surface coatings (Sayes and Warheit 2008) or different crystalline structures (anatase, rutile, or a mixture of both) of TiO₂ particles. The most comprehensive studies aimed at finding a correlation between the characteristics of TiO₂ particles and biological response were those conducted by Sayes et al. (2006), Sayes and Warheit (2008), Simon-Deckers et al. (2008), Braydich-Stolle et al. (2009), and Fujita et al. (2009). These authors tested at least three types of well-characterized TiO₂ particles.

Among the first studies of this type is that by Sayes et al. (2006) in which evidence is provided of dose response on membrane leakage, showing that anatase is more biologically potent than rutile. The same authors provide additional information on the role of surface coatings of nano-TiO₂ in the biological reactivity (Sayes and Warheit 2008). Simon-Deckers et al. (2008) performed an extensive study in which they found only a slight dose-dependent response of some parameters and a time-dependent response which is a function of primary particle size and crystal structure. Braydich-Stolle et al. (2009), however, also report dose-dependent responses but no effects related to primary or secondary particle size and no connection between the biological effect and stability of a suspension (zeta potential). Fujita (Fujita et al. 2009) found no responses dependent on dose, primary particle size, surface area, or particle shape,

Fig. 3 a–c Cell membrane permeability of hepatopancreatic cells after 18 h incubation in suspensions of different types of TiO₂ nanoparticles or Cu²⁺ ions: **a** in 10 nm anatase TiO₂, **b** in 30 nm anatase TiO₂, and **c** in bulk TiO₂. Points on the x-axis represent exposure concentrations (0.1, 1, 10, 100, and 1,000 μg mL⁻¹ of TiO₂ or 100 μg mL⁻¹ Cu²⁺). **p*<0.05; ****p*<0.005 (statistical differences between the control group and exposed groups. *n*—12 gland tubes per concentration (12×200 cells investigated). Symbols on the box plot represent minimum and maximum values (*whiskers*) and mean value (*squares*)

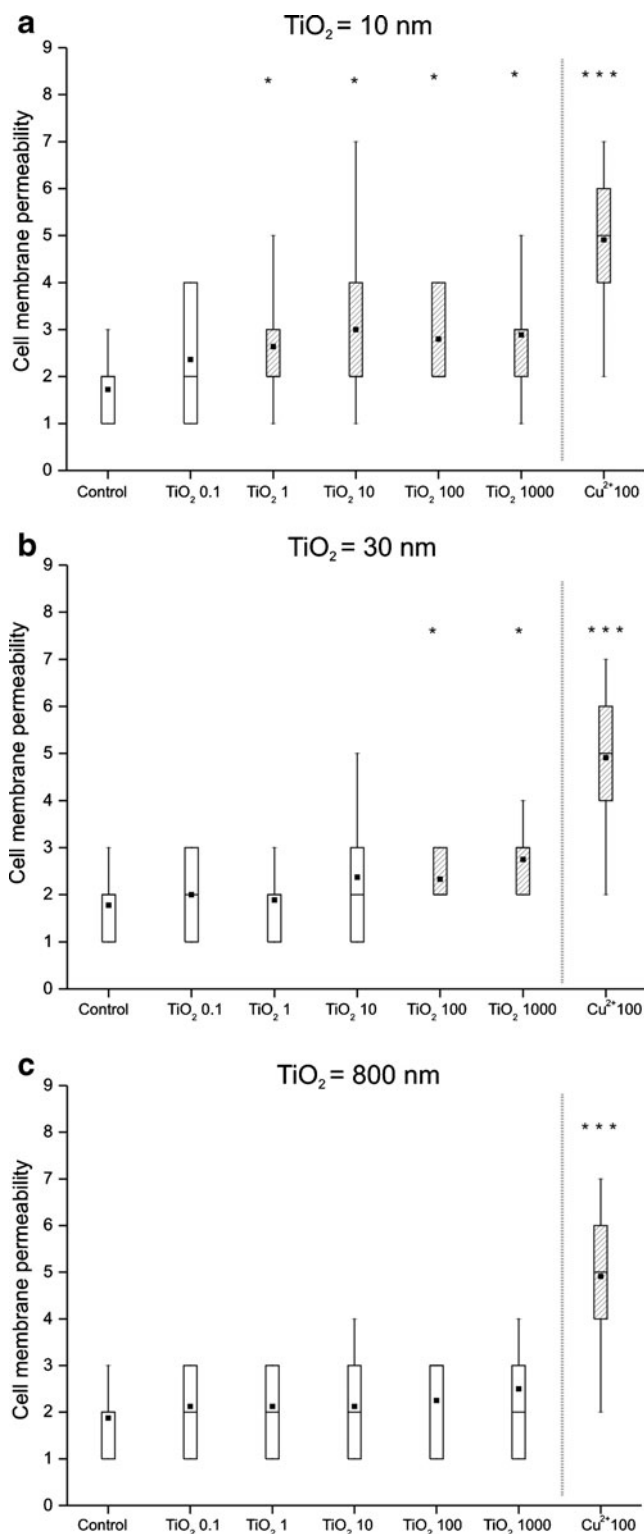
but they recorded a time-dependent response to nano-TiO₂ treatment.

Besides primary particle size, particle surface area, and concentration, shape of nanoparticles can also contribute to their biological reactivity. Some authors found direct connection between particle shape and their ability to penetrate cells. Rod-like nanoparticles were internalized much more rapidly and efficiently than others (Graton et al. 2008).

In numerous reports of correlations between the biological response and the physicochemical characteristics of TiO₂ particles, crystalline structure was generally recognized as a biologically relevant parameter. Some authors report that primary and secondary particle sizes, surface area, duration of exposure, and concentration of particles in a medium are also related to biological effect. It is also clear that the response depends significantly on the biomarker tested as well as the different sensitivity of the cells tested (Sayes et al. 2006). Biomarkers which reflect interaction between nanoparticles and the cell membrane, for example, membrane leakage assessed by a lactate dehydrogenase assay, respond at lower concentrations and earlier than other cellular biomarkers which represent a complex feedback response of cells to stress and include stress defense mechanisms, adverse effects already caused by particles, repair mechanisms, and ultimately, apoptosis or necrosis.

On the basis of these published reports, it is possible to speculate that the effect of TiO₂ particles on biological system may be considered to be time dependent if the exposure times are sufficiently long and dose dependent within specific concentration ranges. Crystalline structure undoubtedly plays a role in biological reactivity and primary particle size and surface area are also relevant. Published reports that the biological reactivity of nanoparticles is not primarily dependent upon particle size and surface area may hinge upon the fact that the compared particles in the tested suspension were significantly modified once suspended.

To prove that the primary size, surface area affect the biological reactivity, we tested two suspensions of nano-sized TiO₂ with similar suspension stability (zeta potential values, -14 and -18 mV) and crystalline form (both anatase) but with different primary particle sizes, surface



area, and confirmed that primary particle size and surface area are related to biological effect. The response was also found to be related to the duration and concentration. More biologically potent particles affected cell membrane at lower concentrations than larger particles (which form larger aggregates). Absence of response of the third particle

type tested in our work, the bulk TiO₂, was either due to the larger size of particles or the presence of the biologically less potent crystalline rutile.

In this study, we provide evidence of the concentration-related threshold response. Increasing the concentration by three orders of magnitude failed to increase the response significantly.

In terms of cell membrane permeability, the lowest observed effective concentration (LOEC) observed in our test system was 1 µg mL⁻¹ for nanosized TiO₂. This is very low compared to other cytotoxicity tests (Thevenot et al. 2008; Simon-Deckers et al. 2008; Vamanu et al. 2008; Sayes and Warheit 2008). For example, when measuring cell membrane stability after exposure of human pneumocytes (A549) to nanosized TiO₂, the LOEC value was more than 20 and 50 µg mL⁻¹ after nonsmall cell lung carcinoma cells (H1299) were exposed to nanosized TiO₂ (Lee et al. 2009; Simon-Deckers et al. 2008). Thus, the ex vivo test applied in our study could be considered to be very sensitive. This is also consistent with the assumption that nanoparticles interact primarily with cell membranes, and only subsequently provoke other cytotoxic responses.

There are also some limitations of ex vivo exposure system. Since digestive glands are isolated tissues, duration of exposure cannot be longer than 18 h. After 18 h, cell death occurs and tissue is not appropriate for analysis.

We conclude that biological potential of nanoparticles is undoubtedly related to their primary size, surface area, shape, and the concentration of nanoparticles in exposure media and duration of exposure. However, many parameters, for example pH of the suspension or presence of other molecules may modify the physiochemical characteristic of particles and make them more or less biologically active than would be suggested by the primary particle characteristics. It is, therefore, necessary to provide a list of characteristics of particles to better understand their biological potential. Even if the particles are well characterized, biological testing is necessary to grasp their biological potential. Two types of data will be required in the future; data on biological potential of nanoparticles and data on nanotoxicity with real relevance for human health and environment. Any of the standard tests can be used to generate data on the biological potential of nanoparticles. Such data could be supplementary to primary particle characteristics and characteristics of particles in a suspension before nanotoxicity studies are started. The ex vivo test system reported here is well suited to the fast screening of the biological potential of nanoparticles. These data may significantly contribute to define further procedures related to particle labeling or further nanotoxicity studies.

Acknowledgments We would like to thank the Slovenian Research Agency (project number J1-9475), Ksenija Kogej and Darko Makovec

for nanoparticles characterization, Živa Pipan-Tkalec and Maja Remškar for TEM analyses, and G.W.A. Milne for editorial assistance.

Conflicts of interest The authors declare that they have no conflict of interest.

References

- Amezaga-Madrid P, Silveyra-Morales R, Cordoba-Fierro L, Nevarez-Moorillon GV, Miki-Yoshida M, Orrantia-Borunda E, Solis FJ (2003) TEM evidence of ultrastructural alteration on *Pseudomonas aeruginosa* by photocatalytic TiO₂ thin films. *J Photoch Photobio* 70(1):45–50
- Banaszak MMH (2009) Nanotoxicology: a personal perspective. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 1(4):353–359
- Braydich-Stolle LK, Schaeublin NM, Murdock RC, Jiang J, Biswas P, Schlager JJ, Hussain SM (2009) Crystal structure mediates mode of cell death in TiO₂ nanotoxicity. *J Nanopart Res* 11(6):1361–1374
- Ferreira NGC, Santos MJG, Domingues I, Calhoa CF, Monteiro M, Amorim MJB, Soares AMVM, Loureiro S (2010) Basal levels of enzymatic biomarkers and energy reserves in *Porcellionides pruinosus*. *Soil Biol Biochem* 42(12):2128–2136. doi:10.1016/j.soilbio.2010.08.008
- Frank SA, Nowak MA (2004) Problems of somatic mutation and cancer. *Bioessays* 26(3):291–299
- Fujita K, Horie M, Kato H, Endoh S, Suzuki M, Nakamura A, Miyauchi A, Yamamoto K, Kinugasa S, Nishio K, Yoshida Y, Iwahashi H, Nakanishi J (2009) Effects of ultrafine TiO₂ particles on gene expression profile in human keratinocytes without illumination: involvement of extracellular matrix and cell adhesion. *Toxicol Lett* 191(2–3):109–117
- Graton SEA, Ropp PA, Pohlhaus PD, Luft JC, Madden VJ, Napier ME, DeSimone JE (2008) The effect of particle design on cellular internalization pathways. *P Natl Acad Sci USA* 105(33):11613–11618
- Hartung T, Daston G (2009) Are in vitro tests suitable for regulatory use? *Toxicol Sci* 111(2):233–237
- Heinlaan M, Ivask A, Blinova I, Dubourguier HC, Kahru A (2008) Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere* 71(7):1308–1316
- Hussain S, Boland S, Baeza-Squiban A, Hamel R, Thomassen LCJ, Martens JA, Billon-Galland MA, Fleury-Feith J, Moisan F, Pairon JC, Marano F (2009) Oxidative stress and proinflammatory effects of carbon black and titanium dioxide nanoparticles: role of particle surface area and internalized amount. *Toxicology* 260(1–3):142–149
- Karlsson HL, Gustafsson J, Cronholm P, Moller L (2009) Size-dependent toxicity of metal oxide particles—a comparison between nano- and micrometer size. *Toxicol Lett* 188(2):112–118
- Lee YS, Yoon S, Yoon HJ, Lee K, Yoon HK, Lee JH, Song CW (2009) Inhibitor of differentiation 1 (Id1) expression attenuates the degree of TiO₂-induced cytotoxicity in H1299 non-small cell lung cancer cells. *Toxicol Lett* 189(3):191–199
- Pan Z, Lee W, Slutsky L, Clark RAF, Pernodet N, Rafailovich MH (2009) Adverse effects of titanium dioxide nanoparticles on human dermal fibroblasts and how to protect cells. *Small* 5(4):511–520
- Park MVDZ, Lankveld DPK, van Loveren H, de Jong WH (2009) The status of in vitro toxicity studies in the risk assessment of nanomaterials. *Nanomedicine* 4(6):669–685
- Ponten J (2001) Cell biology of precancer (Reprinted from *Cancer Surveys* 32, *Precancer Biology, Importance and Possible Prevention*, 1988). *Eur J Cancer* 37:S97–S113

- Poynton HC, Lazorchak JM, Impellitteri CA, Smith ME, Rogers K, Patra M, Hammer KA, Allen HJ, Vulpe CD (2011) Differential gene expression in *Daphnia magna* suggests distinct modes of action and bioavailability for ZnO nanoparticles and Zn ions. *Environ Sci Technol* 45(2):762–768. doi:10.1021/Es102501z
- Regoli F, Nigro M, Orlando E (1998) Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquat Toxicol* 40(4):375–392
- Sayes CM, Warheit DB (2008) An in vitro investigation of the differential cytotoxic responses of human and rat lung epithelial cell lines using TiO₂ nanoparticles. *Int J Nanotechnol* 5(1):15–29
- Sayes CM, Wahi R, Kurian PA, Liu YP, West JL, Ausman KD, Warheit DB, Colvin VL (2006) Correlating nanoscale titania structure with toxicity: A cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. *Toxicol Sci* 92(1):174–185
- Simon-Deckers A, Gouget B, Mayne-L'Hermite M, Herlin-Boime N, Reynaud C, Carriere M (2008) In vitro investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes. *Toxicology* 253(1–3):137–146
- Singh N, Manshian B, Jenkins GJS, Griffiths SM, Williams PM, Maffei TGG, Wright CJ, Doak SH (2009) NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. *Biomaterials* 30(23–24):3891–3914
- Soto K, Garza KM, Murr LE (2007) Cytotoxic effects of aggregated nanomaterials. *Acta Biomater* 3(3):351–358
- Thevenot P, Cho J, Wavhal D, Timmons RB, Tang LP (2008) Surface chemistry influences cancer killing effect of TiO₂ nanoparticles. *Nanomed-Nanotechnol* 4(3):226–236
- Valant J, Drobne D, Sepcic K, Jemec A, Kogej K, Kostanjsek R (2009) Hazardous potential of manufactured nanoparticles identified by in vivo assay. *J Haz Mat* 171(1–3):160–165
- Vamanu CI, Cimpan MR, Hol PJ, Sornes S, Lie SA, Gjerdet NR (2008) Induction of cell death by TiO₂ nanoparticles: Studies on a human monoblastoid cell line. *Toxicol in Vitro* 22(7):1689–1696
- Wang JX, Fan YB, Gao Y, Hu QH, Wang TC (2009) TiO₂ nanoparticles translocation and potential toxicological effect in rats after intraarticular injection. *Biomaterials* 30(27):4590–4600
- Warheit DB (2008) How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? *Toxicol Sci* 101(2):183–185
- Zimmer M (2002) Nutrition in terrestrial isopods (Isopoda: Oniscidea): an evolutionary-ecological approach. *Biol Rev* 77(4):455–493