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CELL MEMBRANE INTEGRITY AND INTERNALIZATION OF INGESTED TIO_2 NANOPARTICLES BY DIGESTIVE GLAND CELLS OF A TERRESTRIAL ISOPOD

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Abstract—The present study was motivated by the paucity of reports on cellular internalization of ingested titanium dioxide (TiO_2) nanoparticles (nano-TiO_2). The model invertebrate (*Porcellio scaber*, Isopoda, Crustacea) was exposed to food dosed with nano-TiO_2 containing 100, 1,000, 2,000, or 5,000 μg nano-TiO_2 per gram of food. After 14 d of exposure, the amount of TiO_2 in the entire body was analyzed by inductively coupled plasma mass spectrometry, and elemental analyses of tissue cross sections were performed by particle induced X-ray emission (PIXE). In addition, a series of toxicological markers including feeding parameters, weight change, and survival, as well as cytotoxic effects such as digestive gland cell membrane stability, were monitored. Internalization of ingested nano-TiO_2 by the isopod's digestive gland epithelial cells was shown to depend on cell membrane integrity. Cell membranes were found to be destabilized by TiO_2 particles, and at higher extracellular concentrations of nano-TiO_2, the nanoparticles were internalized. Environ. Toxicol. Chem. 2012;31:1–9. © 2012 SETAC

Keywords—Nanoparticles TiO_2 *Porcellio scaber* Nanoparticle internalization Membrane stability

INTRODUCTION

Nanomaterials have unique physical and chemical properties as a result of their small particle size, shape, conductivity, and surface chemistry, and consequently they may provoke unique biological responses.

Currently, titanium dioxide (TiO_2) nanoparticles have a wide application in industry and are most commonly encountered among nanoparticles. A consequence is that TiO_2 could become a substantial environmental pollutant. Nanoparticles of TiO_2 have been shown to have different types of effects in vivo [1], although their toxic potential appears not to be very pronounced. Few reports have been published on the distribution and accumulation of TiO_2 in tissues; however, concern exists that bio-accumulated TiO_2 particles may be biomagnified along a food chain and so pose a threat to the ecosystem [2–4].

Federici et al. [5] reported that at exposure concentrations in water of 0.1, 0.5, or 1.0 mg/L TiO_2 nanoparticles for up to 14 d, rainbow trout (*Oncorhynchus mykiss*) manifested respiratory and oxidative stress, organ pathological conditions, and an induction of antioxidant defenses, such as glutathione. However, they were unable to detect accumulation of TiO_2 nanoparticles. Working with rainbow trout and zebrafish (*Danio rerio*), Johnston et al. [6] demonstrated uptake of nano-TiO_2 from the water and small amounts of Ti in gill tissue. The effects and accumulation of ingested TiO_2 nanoparticles on juvenile rainbow trout were further investigated by Ramsden et al. [7], who tested higher concentrations and a longer exposure period than in previous reports [5]. They exposed the fish to diets containing 10 or 100 mg/kg nano-TiO_2 for eight weeks followed by a two-week recovery period and found that the nano-TiO_2 had no impact on growth or nutritional performance. In addition, they observed no major changes in red or white blood cell counts, hematocrits, whole blood hemoglobin, or plasma Na^+; however, accumulation of Ti was observed in the gill, gut, liver, brain, and spleen during this exposure to TiO_2 in the diet. An investigation of the bioaccumulation of TiO_2 nanoparticles in *Daphnia magna* was reported by Zhu et al. [8], who detected particles only in the gut lumen and adduced no evidence of TiO_2 particle internalization. Galloway et al. [9] revealed TiO_2 aggregates of less than 200 nm within the gut lumen in the lugworm *Arenicola marina,* but no uptake of particles across the villi or outer epithelium was observed.

An in vitro study in which accumulation of nano-TiO_2 was investigated using a model system reflecting the components of the digestive system was conducted by Koeneman et al. [10]. Their study provided evidence that, with exposure to levels of TiO_2 above 10 μg/ml, low levels of TiO_2 nanoparticles cross the epithelial lining of the intestinal model by transcytosis. However, the precise mechanism of this transfer remains to be elucidated.

Various in vivo studies have provided data on accumulation of nano-TiO_2 in different organs of experimental animals, but evidence for cellular internalization was sparse and was mostly derived from in vitro experiments or from vertebrate skin studies [10,11]. No in vivo experiments have been reported that provided data on cellular internalization of ingested nano-TiO_2.

The aim of the present study was to examine internalization of ingested nano-TiO_2 by digestive gland epithelial cells of the isopod *Porcellio scaber* (Isopoda, Crustacea). The choice of isopods was motivated by the fact that isopods are exposed to particles in their food and that the consumed amount of particles (actual exposure dose) could be directly linked to any observed

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effect, which is not the case with other invertebrate test organisms. The other advantage of using terrestrial isopods over laboratory vertebrates is that, in contrast to experiments involving animals, use of isopods is not subject to legal restrictions, and in addition, isopods are small enough to allow studies of entire body cross sections by the spectroscopic method selected (particle induced X-ray emission). An attempt is made to relate data on particle assimilation to data on the resulting effects, such as feeding rate, weight change, and survival as well as cytotoxicity to digestive gland cells, that is, cell membrane destabilization.

MATERIALS AND METHODS

Chemicals

Acridine orange, ethidium bromide, trichloroacetic acid, hydrochloric acid, thiobarbituric acid, butylated hydroxytoluene, *n*-butanol, sodium chloride, potassium chloride, magnesium chloride, glucose, and 2-amino-2-hydroxymethyl-propane-1,3-diol, were purchased from Merck. Rhodamine 123, ethanol, and TiO_2 were purchased from Sigma-Aldrich. The TiO_2, which had been used in our earlier experiments [12,13], was supplied as a powder, guaranteed 99.7% pure, with an anatase crystalline structure, average particle size less than 25 nm, and surface area between 200 and 220 m^2/g.

Model organisms

Terrestrial isopods *P. scaber* (Isopoda, Crustacea) were collected during August 2009 at an uncontaminated location near Ljubljana, Slovenia. The animals were kept in a terrarium filled with a layer of moistened soil and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*), at a temperature of $20 \pm 2^{\circ}C$ and a 16:8-h light:dark photoperiod. Only adult animals of both sexes and weighing more than 30 mg were used in the experiments. If molting or the presence of marsupia were observed, the animals were not included in the experiment to keep the investigated population as physiologically and homogenous as possible.

Anatomy of the digestive system of model organism

The digestive system of the terrestrial isopod *P. scaber* is composed of a stomach, four blind-ending digestive gland tubes (hepatopancreas), and a gut (Fig. 1). Food enters the digestive glands directly via a short stomach or after the reflux from the gut, and ingested material is mixed with digestive fluids. Hypothetically, ingested nanoparticles can reach the surface of digestive gland cells immediately after digestion and again after some hours by reflux from the gut.

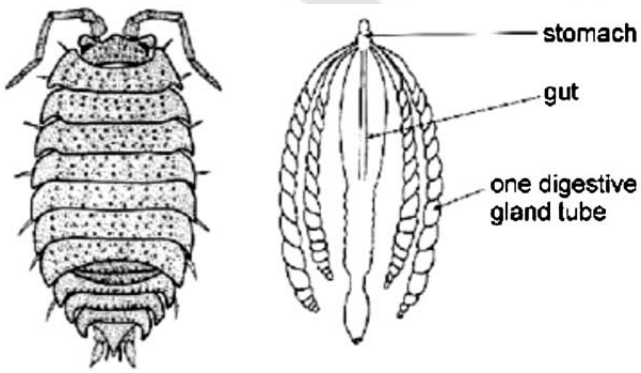

Fig. 1. Sketch of *Porcellio scaber* and digestive system. The stomach, gut, and one tube of digestive gland are marked.

Characterization of nanoparticles

Nanoparticles were inspected with transmission electron microscopy and analyzed by dynamic light scattering, Brunauer-Emmett-Teller, and X-ray powder diffraction techniques. The aim of these analyses was to provide data on the suspension of particles and allow comparisons among different studies and within our experiments.

For transmission electron microscopy, dispersions of nanoparticles (100 μg nano-TiO_2/ml distilled water) were applied on carbon-coated grids, dried at room temperature, examined with a 200 keV field emission transmission electron microscope (Philips CM 100, Koninklijke Philips Electronics), and analyzed by transmission-electron diffraction to determine the TiO_2 phase.

In dynamic light scattering analyses, the dispersions of nanoparticles (100 μg nano-TiO_2/ml distilled water) were inspected using a three-dimensional dynamic light scattering SLS[Q1] spectrometer (LS Instruments). This allows the assessment of the hydrodynamic radii of particles in extremely turbid suspensions by a so-called three-dimensional cross-correlation technique that successfully eliminates multiple scattering of light. 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°. At higher concentrations of nanoparticles (1,000, 2,000, and 5,000 μg/ml), measurements were not possible because of the transparency of the sample.

After the samples were dried and degassed with nitrogen, Brunauer-Emmett-Teller analysis was also applied to TiO_2 samples (Tristar 3000, Micrometrics Co.) to obtain information concerning the surface area of the solid material.

The TiO_2 samples were monitored by X-ray powder diffraction using a Bruker AXS D4 Endeavor diffractometer with Cu-Kα1 radiation and a Sol-X energy dispersive detector within the angular range $20^{\circ} < 2\Theta < 80^{\circ}$, with a step size of 0.04° and a collection time of 3 s.

Food preparation

In the present study, the animals consumed particles applied in a suspension on the leaf surface. Hazelnut leaves were collected in an uncontaminated area and dried at room temperature. Dried leaves were cut into pieces of approximately 100 mg. The TiO_2 nanoparticles were suspended in distilled water before each experiment to obtain different final concentrations (100, 1,000, 3,000, and 5,000 μg/ml).

In the control group, the leaves were treated with distilled water. A suspension of particles or distilled water was brushed onto the lower leaf surface to give final nominal concentrations of nanoparticles on the leaves of 100, 1,000, 3,000, and 5,000 μg nano-TiO_2 per gram (dry wt) of leaf and left until dry.

After exposure, remnants of selected leaves were dried and attached to mounts with silver paint, gold-palladium sputtered (Sputter coater SCD 050, BAL-TEC), and investigated by field emission scanning electron microscopy (SEM) (Jeol JSM-6500F, at the Institute of Metals and Technology). Scanning electron microscopy revealed that particles remained spread over the entire leaf surface (Fig. 2). Energy dispersive X-ray analysis was used to prove their chemical composition (Fig. 2) (EDS/WDS Oxford Instruments INCA, Jeol JSM-6500F, at the Institute of Metals and Technology).

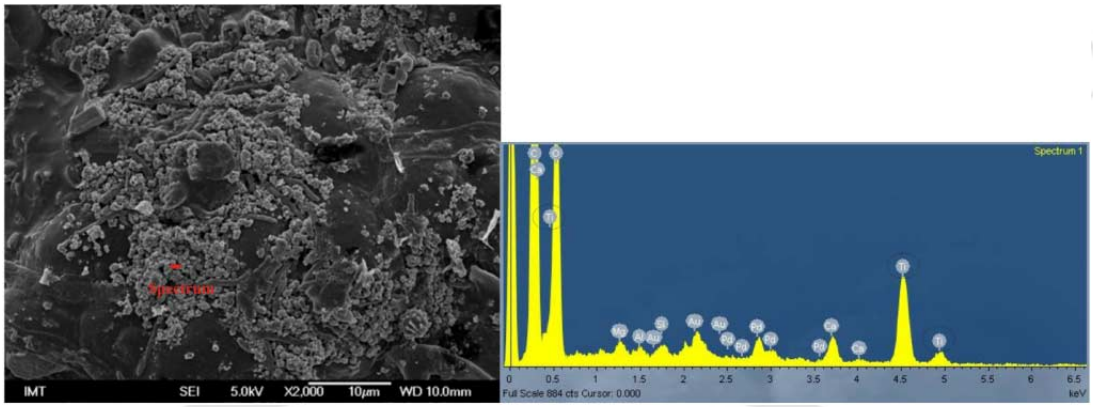

Fig. 2. (**a**) Nano-TiO_2 dispersed over the lower leaf surface to give a final concentration of 5,000 μg/g dry weight leaf. The red spot indicates where the spectrum for energy-dispersive X-ray spectroscopy analysis was performed (EDS) (**b**), to confirm the presence of Ti.

Experimental setup

Each individual animal was placed in a 9-cm Petri dish. One hazelnut leaf treated with distilled water or nano-TiO_2 suspension placed in each Petri dish was the animal's only food source. Humidity in the Petri dish was maintained by spraying tap water on the internal side of the lid every day. All Petri dishes were kept in a large glass container under controlled conditions in terms of air humidity (≥80%), temperature ($21 \pm 1^{\circ}C$), and light regimen (16:8h light:dark photoperiod).

A different number of animals in each individual experiment were exposed to varying concentrations of nanoparticles for 14 d. Four experiments were done (one at a time; experiments A, B, C, and D), as shown in Table 1. The initial number of animals tested was selected on the basis of the type of analyses conducted after exposure.

The concentrations were chosen arbitrarily on the basis provided by our preliminary experiments. After the exposure, the animals were anesthetized at a low temperature and then decapitated and their digestive glands isolated. In different experiments, digestive gland tubes were used for different analyses as detailed in Table 1. In experiments B and C, some whole animals were analyzed. Because data for the control animals from different experiments did not significantly differ statistically, we pooled them together in some results interpretations.

Feeding parameters, weight change, and survival

After 14 d exposure of the animals to treated leaves, the fecal pellets and leaves were removed from the Petri dishes, dried at room temperature for 24 h, and weighed separately. The feeding

Table 1. Fourteen days' dietary exposure studies with *Porcellio scaber*[a]

	Effect measurements		Bioaccumulation and tissue distribution analyses	
Controls and nominal concentrations of nano-TiO_2 on leaves (μg TiO_2/g dry wt of leaves)	Toxicity measurements (feeding parameters, weight change, mortality)	Cytotoxicity measurements (digestive gland cell membrane stability)	Whole body Ti concentrations (IC-PMS)	Tissue distribution and concentration of Ti (PIXE)
Experiment A				(Hepatopancreas)
0 (control)	N = 8	N = 4	—	N = 2
1,000	N = 8	N = 5	—	N = 2
3,000	N = 8	N = 4	—	—
5,000	N = 8	N = 5	—	N = 2
Experiment B				(Whole animals)
0 (control)	N = 13	N = 9	—	N = 1
100	N = 15	N = 10	—	—
1,000	N = 15	N = 8	—	N = 1
5,000	N = 15	N = 8	—	N = 1
Experiment C				
0 (control)	N = 15	—	n = 2	—
1,000	N = 15	—	n = 2	—
3,000	N = 15	—	n = 2	—
5,000	N = 15	—	n = 2	—
Experiment D				
0 (control)	N = 23	N = 12	—	—
1,000	N = 22	N = 18	—	—
3,000	N = 22	N = 16	—	—
5,000	N = 21	N = 18	—	—

[a] Experiments with final nominal exposure concentrations of nano-TiO_2 and parameters measured in this study. In each parameter with different exposure concentrations, a total of *n* animals were analyzed.

IC-PMS = inductively coupled–plasma mass spectrometry; PIXE = particle induced X-ray emission.

rate of isopods was calculated as the mass of consumed leaves per animal's wet weight per day. The food assimilation efficiency was calculated as the difference between the mass of consumed leaves and the mass of fecal pellets divided by the mass of consumed leaf. The amount of TiO_2 particles consumed was calculated on the basis of the quantity of leaf consumed and the amount of TiO_2 particles applied on the leaf, with the assumption that the suspension was applied evenly on the leaf surface. The weight change of an animal was calculated as the difference in its mass from the beginning to the end of the experiment.

Digestive gland cell membrane stability

Cell membrane stability was tested with a modified method for assessment of cell membrane stability, previously described by Valant et al. [12]. A single isolated hepatopancreatic tube was incubated for 5 min in a mixture of the fluorescent dyes acridine orange and ethidium bromide and then put on a microscope slide. Fresh samples were photographed and examined by an Axioimager.Z1 fluorescent microscope (Zeiss) with two different sets of filters. The excitation filter 450 to 490 nm and the emission filter 515 nm (filter set 09) were used to visualize acridine orange- and ethidium bromide-stained nuclei, and the excitation filter 365 nm and the emission filter 397 nm (filter set 01) were used to visualize nuclei stained with ethidium bromide only. Cell membrane integrity was assessed by examination of micrographs. Photographs of intact digestive glands were examined by the same observer twice at intervals of at least 24 h. Cell membrane integrity was assessed visually and classified from 0 to 9 according to a predefined scale. On the basis of preliminary experiments, we concluded that nontreated (control) animals showed less than 5% of nuclei stained by ethidium bromide, whereas severely stressed animals have up to 100% of ethidium bromide-stained nuclei. Less than 5% of the hepatopancreatic tubes stained with ethidium bromide were classified as 0, and those with the highest proportion (>95%) of ethidium bromide-stained nuclei were classified as 9 [12].

Microparticle-induced X-ray emission (micro-PIXE) analysis

For micro-PIXE analysis, digestive glands or whole animals were shock-frozen in liquefied propane or liquid N_2, using tissue-freezing medium (Jung Tissue Freezing Medium, Leica). Samples were sectioned with a thickness of 60 μm using a Leica CM3050 cryotome (Leica) with the temperature of the microtome head and chamber maintained between −25°C and −20°C. The sections were placed in precooled Al holders, transferred to an alpha 2–4 Christ freeze dryer using a cryotransfer assembly cooled with liquid nitrogen, and then freeze-dried at −30°C and 0.4 mbar for 24 h. Dry sections were mounted between two thin layers of Pioloform foil on the Al sample holder [14,15].

For detection of X-rays ranging from 1 keV up to 25 keV, two X-ray detectors were used. A high-purity germanium X-ray detector (active area, 95 mm^2; beryllium window, 25 μm thick; polyimide absorber, 100 μm thick) positioned at 135° to the beam direction was used for the energy range of 4 to 25 keV. Low-energy X-rays in the range of 0.8 to 4 keV were detected by a Si(Li) detector (active area, 10 mm^2) positioned at 125° to the beam direction. The proton dose was determined by a rotating in-beam chopper. Measurement of micro-PIXE and data evaluation for the biological samples of intermediate thickness at the micro-PIXE laboratory at the Jožef Stefan Institute in Ljubljana has previously been described in detail [14,16,17].

In experiment A, sections of two digestive gland tubes from animals fed on food dosed with 1,000 μg nano-TiO_2/g of leaf, two digestive gland tubes from animals fed on food dosed with 5,000 μg nano-TiO_2/g of leaf, and two gland tubes from the control group were analyzed by PIXE. In experiment B, sections of three whole animals were analyzed; one was from the control group, and two were from animals fed on food dosed with 1,000 and 5,000 μg nano-TiO_2/g leaf.

Quantitation of titanium by inductively coupled plasma mass spectrometry

In two parallel experiments, four whole animals from each group were combined, and approximately 30 to 80 mg samples were weighed into microwave digestion quartz vessels. Then, 1 ml 65% HNO_3 (Merck, suprapur) and 1 ml 30% H_2O_2 (Merck, suprapur) were added, and the samples were subjected to closed vessel microwave digestion (Microwave system Ethos 1, Milestone SN 130471) at a maximum power of 1,500 W: ramp to 130°C 10 min, ramp to 200°C 10 min, hold 20 min, cool 20 min. The entire resulting solution was transferred into 10-ml polyethylene graduated tubes and adjusted to 10 ml with ultrapure water (MilliQ system, Millipore). The same procedure was used with blank samples and with NIST 1548a Typical Diet, a certified reference material.

Measurements of the concentrations of elements in digested solutions were made by an Octapole Reaction System Inductively Coupled Plasma Mass Spectrometer (7500ce, Agilent equipped with an ASX-510 Autosampler Cetac). The instrumental conditions used were as follows: nebulizer Micro Mist, spray chamber Scott-type, spray chamber temperature 5°C, plasma gas flow rate 15 L/min, carrier gas flow rate 0.8 L/min, make-up gas flow rate 0.1 L/min, nebulizer pump 0.1 rps, RF power 1,500 W, and reaction cell gases H_2 4 ml/min and He 4 ml/min. Torch position and gas flow rates were optimized daily to give maximum sensitivity. The isotopes monitored were ^{47}Ti and ^{48}Ti, and external calibration was used for quantification.

The certified reference material NIST 1548a Typical Diet was used to check the accuracy of the results. The value measured, 4.2 ± 0.8 μg Ti/g, was in agreement with the value provided by the certified reference material, 4.7 μg Ti/g. The limit of detection was 50 ng/g.

Data analysis

Data were analyzed by standard statistical methods. The difference in the median measured parameters in exposed and unexposed groups was tested with the nonparametric Mann-Whitney *U* test. All calculations were performed with Statgraphics Plus 4.0. Statistical differences between exposed and control animals were divided into three categories with different numbers of asteriks assigned ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Digestive gland cell membrane stability was determined as in our previous work [12] and shown as a percentage of animals per group with different degrees of destabilized cell membrane. The tissue distribution of Ti was shown with elemental distribution maps[Q2] (Fig. 3).

RESULTS

Characteristics of nano-TiO_2

Transmission electron microscopy revealed the shape and size of tested TiO_2 nanoparticles (Fig. 4). The largest particles were elongated spheres whose hydrodynamic radius was shown by dynamic light scattering to be 110 nm. The Brunauer-

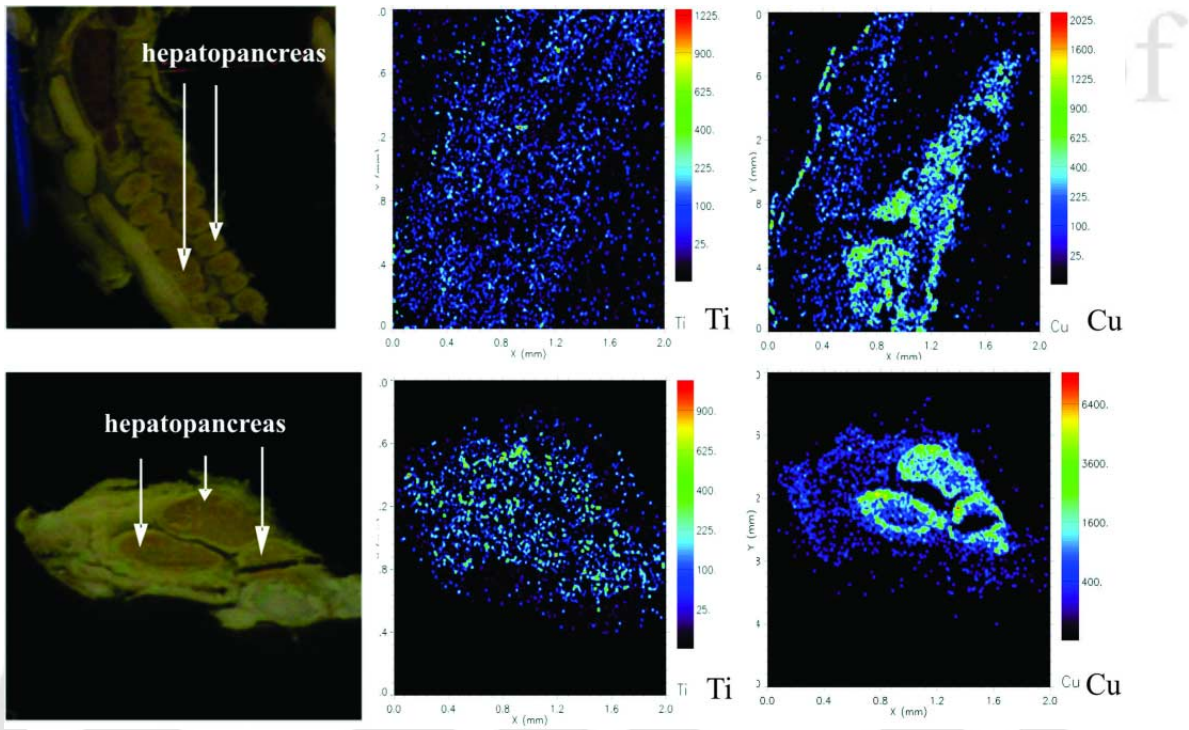

Fig. 3. The first three pictures in the upper row show the longitudinal section of a control animal, distribution map of Ti, and distribution map of Cu. The Cu denotes the location of digestive glands on a section. The lower three pictures show transverse sections of an animal exposed on food dosed with 5,000 μg/g nano TiO_2 dry weight, distribution map of Ti, and distribution map of Cu.

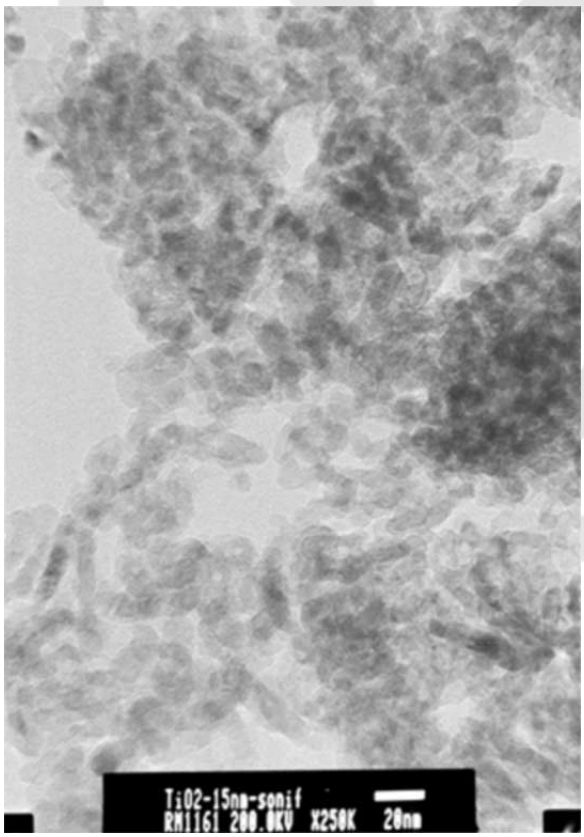

Fig. 4. Transmission electron microscopy of tested nanosized TiO_2 particles, showing their shape and size.

Emmett-Teller method was used to determine the surface area of TiO_2 samples as 144 m^2/g. The size and surface area correspond to the data provided by the supplier. X-ray powder diffraction confirmed that the TiO_2 was in the anatase crystal form.

Effect of ingested nano-TiO_2 on feeding parameters, weight change, and survival

Animals were exposed to leaves dosed with nano-TiO_2 suspension, providing nominal concentrations of 100, 1,000, 3,000, or 5,000 μg nano-TiO_2/g of leaf. Feeding parameters, weight, and survival were not affected below a nominal exposure concentration of 5,000 μg nano-TiO_2/g dry weight of leaf (Table 2). Feeding rate was approximately 0.05 mg food/mg animal weight/day, and no statistical differences were found between animals in the control group and those that were exposed. Based on the amount of food consumed in 14 d, animals ingested approximately 0.2 ± 0.1 μg TiO_2 per day when fed on leaves with 100 μg nano-TiO_2/g, 2.1 ± 0.7 μg TiO_2 per day when fed on leaves with 1,000 μg nano-TiO_2/g, 6.0 ± 2.1 μg TiO_2 per day when fed on leaves with 2,000 μg nano-TiO_2/g, and 9.8 ± 3.5 μg TiO_2 per day when fed leaves with 5,000 μg nano-TiO_2/g.

Effect of ingested nano-TiO_2 on digestive gland cell membrane stability

Our previously published data demonstrate that in animals from a stock culture, which are in good physiological condition, the digestive gland cell membrane stability value was rarely higher than 2, and this was taken as a benchmark [12]. The higher the value the more the membrane is destabilized, and the cell membranes are considered completely destabilized when the value is higher than 2.

Table 2. Lowest observed effect concentration (LOEC), lowest observed effect dose (LOED), and no observed effect concentration (NOEC) of nano-TiO_2 that affected the measured parameters in 14-d dietary exposure studies with *Porcellio scaber*

	Effects of ingested nano-TiO_2		Body concentration and tissue distribution of Ti	
	Feeding parameters, weight change, and survival	Digestive gland cell membrane stability	Concentration of Ti in entire organism	Tissue distribution and concentration of Ti
Effect concentrations	LOEC 5,000 μg/g nano TiO_2 in the food; NOEC 100, 1,000, 3,000 μg/g nano TiO_2 in the food	LOEC 1.000 μg/g nano TiO_2 in the food; LOED 2.5 μg TiO_2 per day; NOEC 100 μg/g nano TiO_2 in the food	Ti accumulated in only some animals at 5,000 μg/g	Intracellular deposition found in animals with destabilized cell membrane at 5,000 μg/g nano TiO_2 in the food

In control animals and those exposed to food with the lowest amount of nano-TiO_2 (100 μg/g), cell membranes were not affected in more than 10% of the animals. However, an exposure concentration of 1,000 μg nano-TiO_2/g in the food caused digestive gland cell membrane destabilization in up to 39% of exposed animals (Fig. 5). The pattern of cell membrane destabilization was not dose-dependent. The highest proportion of animals with destabilized membranes was found in a group fed on food dosed with 1,000 μg TiO_2/g (39% of the animals) and 5,000 μg TiO_2/g (32%).

Effect of ingested nano-TiO_2 on whole body Ti concentrations

No differences were found in the concentration of Ti in whole animals in the control group and those fed on food dosed with 1,000 or 3,000 μg nano-TiO_2/g leaf (Table 3). In animals fed on food dosed with 5,000 μg TiO_2/g leaf, significantly higher Ti content was found in one pooled sample of four animals when compared with a control group, but in the other pooled sample from the same experimental group the Ti concentration was much lower.

Because significant amounts of Ti were detected only in some animals (Table 3), this suggests that when animals are fed on food dosed with 5,000 μg TiO_2/g dry weight of leaf, Ti is either deposited on the cell surface or remains in the lumen of the digestive system without being assimilated by cells. To confirm the presence of Ti inside cells, tissue cross sections were investigated by the PIXE method.

Tissue distribution and concentration of Ti

In whole body sections of one control and two animals fed on food dosed with nano-TiO_2 at 1,000 μg/g and 5,000 μg/g nano-TiO_2 in dry weight of food and subsequently analyzed by PIXE, titanium was not detected in any tissue (Fig. 3). Parallel to Ti, we also analyzed the distribution of Cu, which is used to show the content of digestive gland epithelium because co-localization of Cu and Ti would indicate that Ti is present in the digestive gland epithelium.

Additional PIXE analyses were performed on cross sections of the isolated digestive gland tubes (Fig. 6) of animals fed on food containing 1,000 or 5,000 μg/g nano-TiO_2. In all animals with an unaffected cell membrane and a digestive gland cell membrane stability value 1 (Table 4) and fed with food dosed with either 1,000 or 5,000 μg nano-TiO_2/g of leaf, traces of Ti were detected in the digestive gland epithelium (Table 4). However, a substantial amount of Ti was found in the digestive

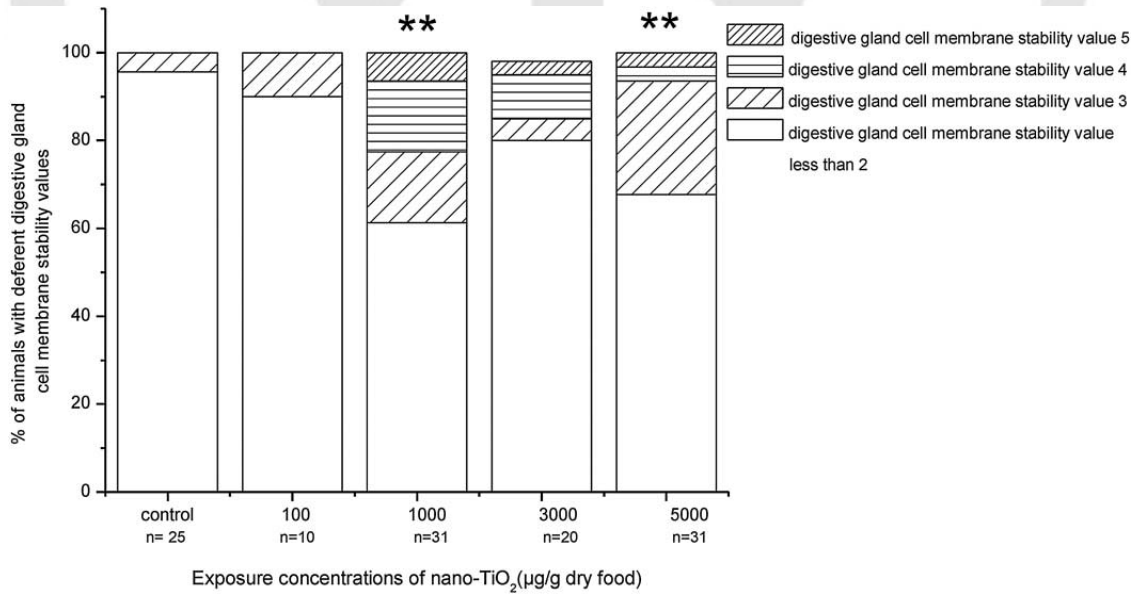

Fig. 5. Percentage of animals in each group with different degrees of destabilized cell membrane, assessed visually and classified from 0 to 5 according to the predefined scale as described in Materials and Methods. Data[Q3] from experiments A, B, and D are pooled together. Digestive gland cell membrane stability value of 2 or less denotes animals that did not have destabilized cell membranes and digestive gland cell membrane stability values from 3 to 5 animals with destabilized cell membranes. The value of 5 corresponds to the most highly destabilized cell membranes. Statistical differences between exposed and control animals were categorized into three groups, with different numbers of asterisks assigned ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Data from experiments A, B, and D were pooled.

Table 3. Concentration of Ti (ng/g) in whole animals fed on food dosed with nano-TiO_2 for 14 days measured with inductively coupled plasma mass spectroscopy (ICP-MS)[a]

Samples	No. of samples	Ti concentration (μg/g)	Mean value (μg/g)	Absolute error (μg/g)
Control	2	9.3		
	2	8.7	9.0	0.3
TiO_2 1,000	2	9.3		
	2	11.5	10.4	1.1
TiO_2 3,000	2	12.7		
	2	9.5	11.1	1.6
TiO_2 5,000	2	12.1		
	2	30.2	21.2	9.1

[a] n represents two parallel measurements in which two samples of four pooled whole animal samples were taken; altogether 8 animals (TiO_2 1,000—animals fed on food dosed with 1,000 μg nano-TiO_2/g of leaf; TiO_2 3,000—animals fed on food dosed with 3,000 μg nano-TiO_2/g of leaf; and TiO_2 5,000—animals fed on food dosed with 5,000 μg nano-TiO_2/g of leaf).

gland epithelium of animals fed on 5,000 μg nano-TiO_2/g of leaf with destabilized digestive gland cell membranes.

DISCUSSION

Our results confirm internalization of ingested nano-TiO_2 by digestive gland epithelial cells of the terrestrial isopod *P. scaber* as a result of compromised cell membrane integrity. However, Ti was detected in digestive gland epithelial cells only when both the exposure dose was high (9.8 ± 3.5 μg TiO_2 per day for 14 d) and the digestive gland cell membrane was destabilized. At some doses cell membrane destabilization was observed, no evidence was found of organism level toxicity responses. To our knowledge, no other in vivo evidence exists on cellular internalization of ingested nano-TiO_2 coupled to toxicity data.

Nano-TiO_2 exposure concentration up to 5,000 μg nano-TiO_2/g of leaf resulted in no toxicity to the terrestrial isopod *P. scaber* when measured by feeding parameters, weight change, or survival. However, when cytotoxicity is taken as a measure, 1,000 μg/g nano-TiO_2 in the food for 14 d destabilized cell membranes in approximately 30% of the animals. This response was not exposure concentration related.

Abe et al. [18] studied the internal diffusion and absorption of TiO_2 particles through the digestive system of mice and reported that TiO_2 particles fed to mice were detected in the lung, liver, and spleen after 10 d of exposure. They discovered that, compared with intravenous injection, the absorption of orally ingested TiO_2 was extremely low. Biodistribution of TiO_2 nanoparticles administered to mice as a single oral gavage has been studied by Wang et al. [19], who reported that TiO_2 particles are transported into other tissues and organs via the gastrointestinal tract after uptake, and induce significant lesions, particularly of the liver and kidneys. The TiO_2 nanoparticles were found by this group to have a wide tissue distribution being found even in the brain. Our findings on low internalization of ingested nano-TiO_2 are in line with these reports. However, the effect of chronic exposure to TiO_2 nanoparticles remains to be investigated. Cell membrane injury in invertebrates may possibly lead to the cellular internalization of TiO_2 particles and potential distribution of particles into other organs.

Simultaneous toxicity and cellular internalization of ingested nano-TiO_2 by environmental organisms have not been previously investigated in in vivo experiments. In the present study, we employed micro-PIXE to document the presence of Ti inside digestive gland epithelial cells. A sample region of 2,000 μm × 2,000 μm × 60 μm was analyzed, and the main advantages of this method are high elemental sensitivity and low lateral resolution in the micron range. In addition, for biological samples, sample preparation must involve no exogenous chemicals [14]. Micro-PIXE has also been successfully used in skin penetration studies of nano-TiO_2 [20] and other particles [21].

Our results show that ingested nano-TiO_2 is not internalized if the digestive gland cells remain intact. Adachi et al. [11] reported that no TiO_2 particles could be found in viable skin, and the findings of Sadrieh et al. [22] also indicate no significant penetration of TiO_2 nanoparticles through the intact normal epidermis. We have shown that Ti can be internalized but not before nano-TiO_2 affects the cell membrane, and so internalization can be viewed as proceeding by a two-step mechanism. First, the TiO_2 destabilizes the cell membrane, and in the second step, it is internalized. When the extracellular concentration of particles is sufficiently high, Ti can also be detected intracellularly. In the present study, the exposure concentration that shows this effect was 5,000 μg/g nano-TiO_2 in the food, corresponding to 12 μg TiO_2 per day for 14 d or 0.3 μg TiO_2 per day/mg wet weight of animal in 14 d.

In light of these results, we can explain the findings of Adachi et al. [11] and Sadrieh et al. [22]. The exposure concentrations in their experiments were too low to cause cell membrane destabilization and subsequent cellular internalization.

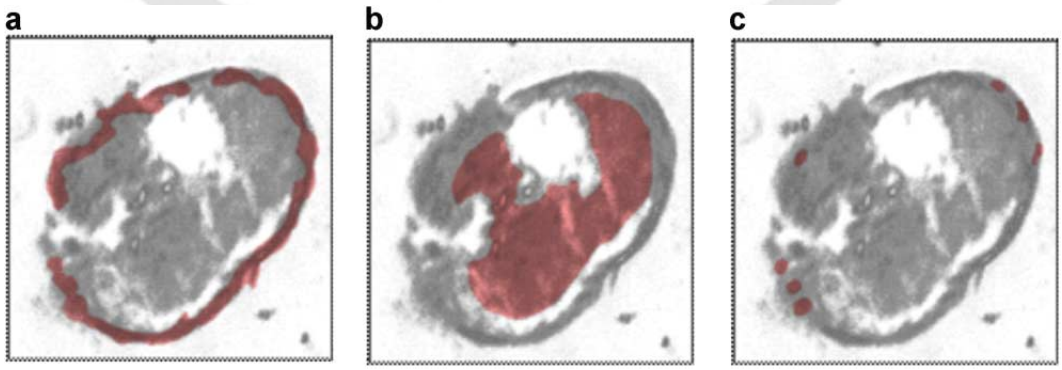

Fig. 6. Areas on cross section of digestive glands marked in red are where measurements of Ti with particle-induced X-ray emission (PIXE) were performed in the following areas: (**a**) digestive gland epithelium, (**b**) digestive gland lumen, and (**c**) selected cells on the digestive gland epithelium.

Table 4. Concentrations of Ti (μg/g) in digestive gland epithelium, lumen, and cells analyzed by particle induced X-ray emission (PIXE) in control and animals fed with nano-TiO_2 for 14 d and corresponding degree of digestive gland cell membrane destabilization presented as digestive gland cell membrane stability value[a]

Sample	Concentration of Ti in digestive gland epithelium (μg/g)	LOD (μg/g)	Concentration of Ti in digestive gland lumen (μg/g)	LOD (μg/g)	Concentration of Ti in digestive gland cells (μg/g)	LOD (μg/g)	Digestive gland cell membrane destabilisation value
C/1	ND	20	ND	15	ND	53	1
1,000/1	ND	5	4.9	3	ND	19	1
1,000/2	8.4	3	$\leq$ lod	4	10.4	7	3
5,000/1	57.4	4	70.6	4	34.4	12	3
5,000/2	4.3	4	4.5	4	ND	12	1

[a] TiO_2 1,000 = animals fed on food dosed with 1,000 μg nano-TiO_2/g of leaf; TiO_2 5,000 = animals fed on food dosed with 5,000 μg nano-TiO_2/g of leaf; number 1 or 2 denotes different samples.
C = control; LOD = limit of detection.

CONCLUSIONS

First, at nominal exposure concentrations ranging from 100 to 5,000 μg/g of nano-TiO_2 in the food, no effects on feeding behavior, weight change, or survival were evidenced in the terrestrial crustacean *P. scaber* after 14 d feeding on TiO_2 - dosed food. Second, cell membrane destabilization in digestive gland cells was observed in approximately 30% of animals fed on food dosed with 1,000 or 5,000 μg nano-TiO_2/g. This effect was apparently not exposure concentration-related. Last, cellular internalization of Ti was found when the following two conditions were both met: when exposure concentration was at least 5,000 μg/g nano-TiO_2 in the food, and when the cell membrane was destabilized.

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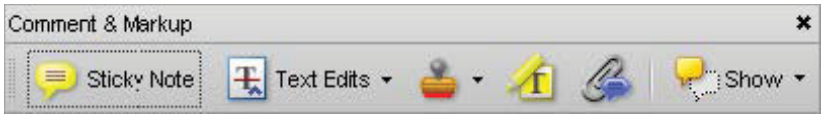

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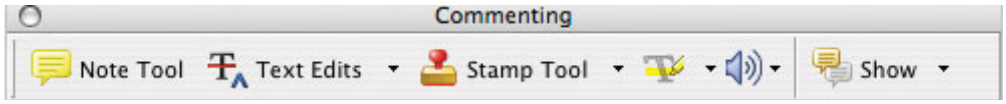

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