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Hazardous potential of manufactured nanoparticles identified by *in vivo* assay

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ABSTRACT

New products of nanotechnologies, including nanoparticles, need to be assessed according to their biological reactivity and toxic potential. Given the large number of diverse nanomaterials, a tiered approach is favoured. The aim of our work presented here is to elaborate an *in vivo* assay with terrestrial invertebrates (*Porcellio scaber*), which could serve as a first step of hazard identification of nanoparticles. We adapted the widely used acridine orange/ethidium bromide (AO/EB) assay to be applicable for cell membrane stability assessment of entire organ where the animal was exposed *in vivo*. The digestive glands (hepatopancreas) of terrestrial isopods were taken as a model test system. The assay was validated with $Cu(NO_3)_2$ and surfactants. The results showed that all tested nanoparticles, i.e. nanosized TiO_2, nanosized ZnO and fullerenes (C_{60}) have cell membrane destabilization potential. As expected, C_{60} is the most biologically potent. The AO/EB *in vivo* assay proved to be fast because response is recorded after 30 min of exposure, relatively simple because digestive glands are inspected immediately after isolation from exposed animals and promising approach because different types of nanoparticles could be tested for their biological potential. This assay provides data for the identification of hazardous potential of nanoparticles before subsequent steps in a tiered approach are decided.

1. Introduction

Nanoparticles differ substantially from bulk materials having the same composition. Novel properties distinguishing nanoparticles from the bulk material typically become apparent at critical particle lengths below 100 nm. Particles of this size have numerous potential technological applications [1] but may be hazardous as a result of a variety of interactions with biological systems possibly leading to harmful effects.

Much evidence suggests that nanoparticles can effectively disrupt cell membranes by nanoscale hole formation, membrane thinning [2], and lipid peroxidation. Recent papers report *in vivo* and *in vitro* [3] effects of nanoparticles on membrane stability. Membrane permeabilization by nanoparticles was proven by enzyme leakage systems [4] and dye diffusion studies [2]. Nanosized titanium dioxide and zinc oxide are known to disrupt cell membrane stability as a result of their photocatalytic properties [5]. A colloidal suspension of nano-C_{60} disrupts normal cellular function through lipid peroxidation; reactive oxygen species (ROS) are responsible for damage to the membrane and its destabilization [6]. An appealing method for assessing cell membrane integrity employs acridine orange/ethidium bromide (AO/EB) and is widely conducted in *in vitro* studies with different types of cells. This paper describes a modification of the AO/EB method for the *in vivo* study of potentially toxic interactions between nanoparticles and biological systems.

The AO/EB assay is based on the assumption that changes in cell membrane integrity result in differences in permeability of cells to AO and EB dyes. Different permeability by the two dyes results in differentially stained nuclei as follows. Acridine orange is taken up by cells with membranes that are intact or destabilized, and in the cell, emits green fluorescence, as a result of its intercalation into double-stranded nucleic acids. Ethidium bromide on the other hand, is taken up only by cells with destabilized cell membranes, and it emits orange fluorescence, after intercalation into DNA [7]. Spectroscopy is used to determine the difference between green and orange emissions, and this provides a measure of cell membrane destabilization. The assay has been applied to a variety of medical [8], pharmacological [9], biotechnological [10] and cell biology [11] studies.

Contrary to other studies, in our work the AO/EB assay was modified to test membrane destabilization of cells in a single-layer epithelium, i.e. digestive glands of the isopod *Porcellio scaber*. Here we assess the membrane destabilization potential of particles and chemicals to which the organism is exposed.

Terrestrial isopods are probably among the best choice for toxicity studies under controlled laboratory conditions. They fulfill most of the criteria to be suitable test organism [12]. Their biology and physiology are well-known, it is relatively easy to maintain a lab-

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oratory culture and handle individual animals in the experiment, and above all it is possible to acquire individual toxicity data at different levels of biological complexity (cell, tissue and organism) [13]. *P. scaber* was successfully used in detecting effects of variety of chemical pollutants [14] and manufactured nanoparticles [15,16]. In addition, terrestrial isopods as invertebrates are not subjected to the same legal restrictions related to animal experimentation as vertebrates.

The first aim of our work was to modify the AO/EB method to be applicable also for *in vivo* analyses. The method was validated using two positive controls with known adverse effects on membranes: copper ions (Cu^{2+}) and surfactants. Excessive concentrations of Cu^{2+} promote formation of reactive oxygen species which can damage membranes by promoting lipid peroxidation, leading finally to increased permeability of the cell membrane [17]. Two surfactants, saponin and polymeric alkylpyridinium salts (poly-APS), are known to directly affect the permeability of the cell membrane. Saponins are natural surfactants found in many plants and are able to disrupt cell membranes through cholesterol binding [18]. Polymeric alkylpyridinium salts are surfactant-like compounds isolated from the Adriatic marine sponge *Reniera sarai* [19], and have cytotoxic [20], haemolytic [21], and antitumour activities [22].

The second aim of this work was to determine the cell membrane destabilization potential of nanosized ZnO, fullerenes, TiO_2 and bulk ZnO after oral exposure to model organism, *P. scaber*. We expected that nanoparticles would alter the stability of the cell membrane resulting in its destabilization and permeability to fluorescent dyes. We also expected that the size of nanoparticles would play an important role in cell membrane destabilization; smaller particles were expected to be more biologically potent. We discuss the suitability of this *in vivo* test system to identify cell membrane destabilization potential of nanoparticles. We also discuss the use of these results for deciding subsequent steps in testing safety of nanoparticles.

2. Materials and methods

2.1. Chemicals

The test chemicals were dissolved in a physiological solution modified for *P. scaber* (248 mM NaCl, 8 mM KCl, 5 mM $MgCl_2$, 5 mM glucose and 10 mM Tris in bidistilled water) [23]. Acridine orange (AO), ethidium bromide (EB), saponin and $Cu(NO_3)_2$, a source of Cu^{2+} ions, were purchased by Merck (Darmstadt, Germany). The concentration of Cu^{2+} ions in the stock solution was determined by atomic absorption spectroscopy (AAnalyst 100, PerkinElmer) and was within 10% of the expected concentration. Poly-APS were purified from the marine sponge *R. sarai* as described previously [19,20], lyophilized and kept frozen until use. A stock solution prepared by dissolving 10 mg of lyophilized poly-APS in 1 ml of the physiologic solution was kept at 4 °C prior to use. Aqueous solutions of poly-APS are known to be stable for years when kept in this way (K. Sepčić, personal communication). Prior to the test, the stock solution was diluted appropriately in physiologic solution to obtain the desired final concentration of the compound.

Nanosized and bulk ZnO, fullerenes, and TiO_2 were purchased (Sigma–Aldrich, Steinheim, Germany). The TiO_2 was supplied as a powder, anatase crystalline structure, average particle size <25 nm and surface area between 200 and 220 m^2/g with guaranteed 99.7% purity as described in our previous experiments [15]. The characteristics of nanosized ZnO and fullerenes were determined as described below. Titanium dioxide and ZnO were suspended in a physiologic solution and distilled water to observe the effect of physiologic solution on particle agglomeration. The solution of fullerenes was prepared only in distilled water.

We tested both bath sonicated (30 min, 20 kHz) and non-sonicated nanosized TiO_2 and ZnO. Only sonicated fullerenes were used in our assays.

Nanosized TiO_2 and nanosized ZnO were not photo activated by UV and near UV light. All solutions were kept protected against UV light.

2.2. Animals

Individuals of the terrestrial isopod *P. scaber* (Isopoda, Crustacea) were collected during August 2006 at a non-contaminated location near Ljubljana, Slovenia. The animals were kept at a temperature of 20 ± 2 °C and a 16-h:8-h light:dark photoperiod and were fed with hazelnut leaves (*Corylus avellana*). Only adult animals exceeding 30 mg were used in the experiments. If moulting or the presence of marsupia was noted, the animals were eliminated from the experiments to keep the investigated population as homogenous as possible in terms of their physiological state. During dissection, the digestive glands were isolated, and their colour and shape were recorded.

2.3. Characterisation of nanoparticles

We used transmission electron microscopy (TEM), dynamic light scattering (DLS) and Braunauer–Emmett–Teller (BET) analysis [24] for characterisation of nanoparticles tested in our study. For TEM, dispersions of all tested nanoparticles were applied on carbon-coated grids, dried at room temperature and examined with a 200 keV field emission transmission electron microscope (Philips CM 100, Koninklijke Philips Electronics, Eindhoven, The Netherlands).

The dispersions of nanoparticles (100 μg/ml) were inspected by DLS using a 3D DLS-SLS spectrometer (LS Instruments, Fribourg, Switzerland). The instrument enables the determination of hydrodynamic radii of particles in extremely turbid suspensions by a so-called 3D cross-correlation technique [25] that successfully eliminates multiple scattering of light. As the light source a HeNe laser operating at a wavelength of 632.8 nm was used. Scattering was measured at an angle of 90°.

All dispersions were analyzed using BET analysis (Tristar 3000, Micrometrics Co., Norcross, GA, USA) to obtain information about the surface area of solid material. Samples were dried and degassed with nitrogen prior to analysis. The limitation of the instrument is that relatively large amounts of material (50 mg) are required for analysis.

2.4. Experimental setup: AO/EB staining

The AO/EB dyes were applied along with the solutions of the chemicals directly on animal's mouth. The volume of the staining solution applied to animal's mouth (in μl) was 1/10 of the animal's weight; a 30 mg animal received 3 μl of solution. This procedure was repeated three times, at intervals of 10 min. Dissection began 10 min after the last application of the AO/EB mixture. This exposure protocol was elaborated on the basis outcome of the preliminary studies, where we applied vital dyes (methylene blue or neutral red) on animal's mouth and inspected the distribution of the dye within animal's digestive system. After oral application of a solution, we could not assess the actual amount of solution that entered the digestive system. A minor portion remained in the region of mouthparts. However the majority of the solution entered the digestive system. This is a novel dosing procedure for *P. scaber* which appears more controllable as substratum or food exposure which are usually used.

Six animals were exposed to each concentration of Cu^{2+}, each concentration of surfactants and each concentration of nanoparti-

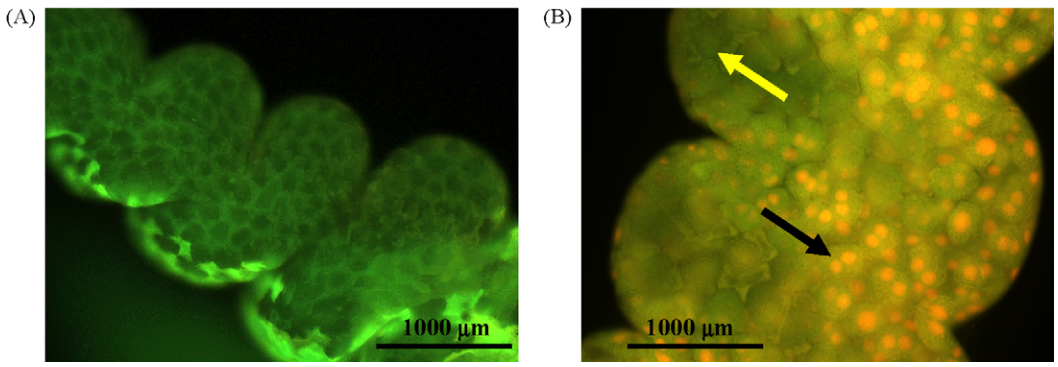

Fig. 1. Micrographs of hepatopancreatic tissue of *P. scaber* taken by fluorescent microscope. (A) Negative control, no nuclei are stained with EB and (B) positive control, cells with destabilized membranes have nuclei stained with EB (orange). Yellow arrow on the figure B represents negative nuclei (not stained with EB) and black arrow represents positive nuclei (stained with EB). (For interpretation of the references to the colour in this figure legend, the reader is referred to the web version of the article.)

cles. All together in the entire experiment 18 × 6 were sacrificed. Animals of both sexes were used.

2.5. Preparation of the staining solutions for positive and control treatments

2.5.1. Control treatment

In control experiments, 10 μl of AO and 10 μl of EB were mixed with 20 μl of the physiological solution.

2.5.2. Oral application of Cu^{2+}

In the test experiment, 10 μl of $Cu(NO_3)_2$ solution was mixed with 10 μl of AO and 10 μl of EB and 10 μl of the physiological solution was applied to the animal's mouth. The final concentration of $Cu(NO_3)_2$ was 1, 10, 100, or 1000 μg/ml.

2.5.3. Oral applications of saponin and poly-APS

In the oral application of saponin and poly-APS, 20 μl of the saponin (final concentrations 0.0005, 50 and 200 mg/ml) or poly-APS (final concentrations 0.022, 1.1 and 2.2 mg/ml) in each case were mixed with 10 μl of AO and 10 μl of EB. These concentrations were chosen on the basis of preliminary experiments.

2.6. Exposure to particles

2.6.1. Oral application of nanoparticles or bulk material

Twenty microliters of nanoparticles or bulk material (final concentration 1000 μg/ml) were mixed with 10 μl of AO and 10 μl of EB and 10 μl of physiologic solution.

2.7. Fluorescence microscopy

Two digestive gland tubes were put on a microscope slide immediately after isolation, and were photographed by an Axioimager.Z1 fluorescent microscope (Zeiss) using two different sets of filters (Zeiss, Axioimager.Z1). The excitation filter 450–490 nm and the emission filter 515 nm (filter set 09) were used to visualize AO and EB stained nuclei, while the excitation filter 365 nm and the emission filter 397 nm (filter set 01) were used to visualize nuclei stained with EB only.

2.8. Generation of a ranking scale

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 the predefined scale. On the basis of preliminary experiments, we concluded that non-treated (control) animals showed <5% of nuclei stained by EB, while severely stressed animals have up to 100% of stained nuclei. The <5% of hepatopancreatic tubes stained with EB were classified as 0, and those with the highest portion (>95%) of EB stained nuclei as 9 (Fig. 1).

2.9. Statistical analysis

Data were analyzed using standard statistical methods. The difference of the medians of measured parameters in exposed and unexposed groups was tested applying the non-parametric Mann–Whitney *U*-test. All calculations were done using STATGRAPHICS Plus 4.0 statistics software for Windows. Statistical differences between exposed and control animals were categorised into three groups with different numbers of stars assigned ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Characterisation of nanoparticles

Transmission electron microscopy reveals the shape, size and aggregation pattern of nanoparticles tested in our study (Fig. 2). No significant differences in the TEM images were observed if nanoparticles were suspended in physiological solution or in distilled water.

Dynamic light scattering analysis was used to determine the size of aggregates of tested suspensions of nanosized TiO_2 and ZnO, bulk ZnO (sonicated and non-sonicated) and fullerenes (sonicated) in distilled water and physiologic solution. The concentration of all nanoparticles investigated by DLS was 100 μg/ml. We were unable to investigate high concentrations of nanoparticles by this method (1000 μg/ml) due to very rapid sedimentation of particles and large polydispersities of samples. Consequently suspensions were diluted by a factor of 10 before analysis.

We have observed differences between sonicated and non-sonicated samples in values obtained with DLS. The average value of the hydrodynamic radius, R_h, of TiO_2 particles in sonicated aqueous solution (100 μg/ml) was 420 nm and in non-sonicated dispersion 870 nm. The average size of ZnO particles (100 μg/ml) was 610 and 740 nm in sonicated and non-sonicated dispersions, respectively, whereas for sonicated fullerene particles (100 μg/ml) R_h values were around 180 nm and the polydispersion was fairly low. The average particle size of nanosized TiO_2 and ZnO suspended in physiological solution could not be measured accurately because of the presence of many large agglomerates probably with sizes larger than the detection limit of the instrument (over 1.5 μm).

The Braunauer–Emmett–Teller (BET) determined surface area of nanoparticles tested in our study was 144 m^2/g for TiO_2 and 12.1 m^2/g for ZnO. Thus the nanoparticle size of the TiO_2 was 10 nm and that of ZnO was 85 nm. The surface area of C_{60} was not determined due to the limited amount of material available.

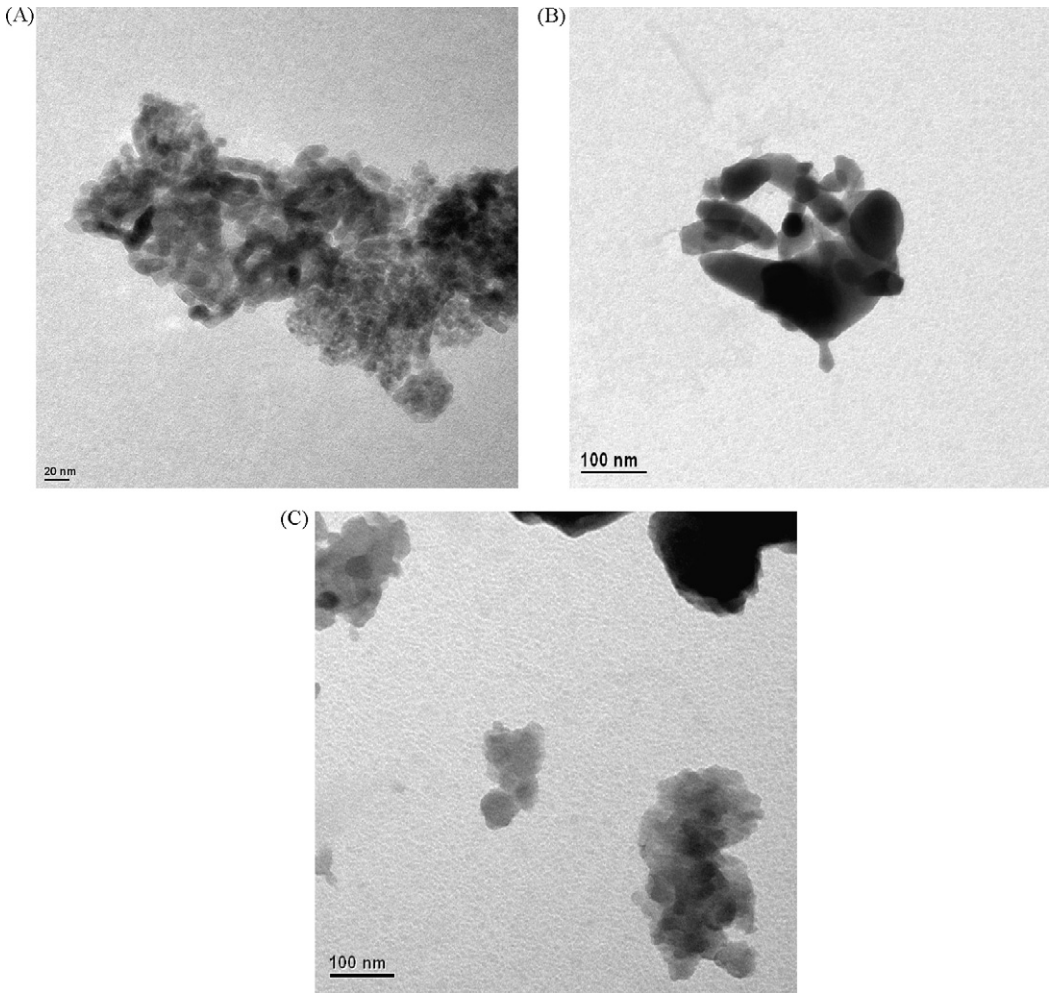

Fig. 2. TEM images of nanosized TiO_2 (A); ZnO (B) and C_{60} (C).

3.2. Effect of ions and surfactants on membrane stability

3.2.1. Oral application of Cu^{2+} and AO/EB staining

After oral application of Cu^{2+}, the membrane integrity of hepatopancreatic cells was clearly compromised in comparison to that of controls (Fig. 3). In control animals, few or none were stained with EB. In animals to which 1, 10, 100, and 1000 μg/ml of Cu^{2+}, was orally applied, the cell membrane permeability was increased. Statistically significant differences between control and Cu^{2+} treated animals were observed when 10, 100 or 1000 μg/ml of Cu^{2+} was applied orally.

3.2.2. Oral application of saponin and poly-APS

Destabilization of the cell membrane was accomplished by oral application of different concentrations of saponin and poly-APS (Fig. 3). The lowest concentrations of saponin (0.0005 mg/ml) and poly-APS (0. 022 mg/ml) tested did not cause significant changes in cell membrane permeability. In animals orally exposed to 50 or 200 mg/ml of saponin and 1.1 or 2.2 mg/ml of poly-APS, the cell membrane permeability changed significantly.

3.3. Effect of nanoparticles on membrane stability

3.3.1. Oral application of nanosized TiO_2, ZnO and C_{60}

The ability of nanosized TiO_2 or ZnO to destabilize the cell membrane *in vivo* was tested by oral application of sonicated and non-sonicated suspensions. Results of oral application of nanopar-

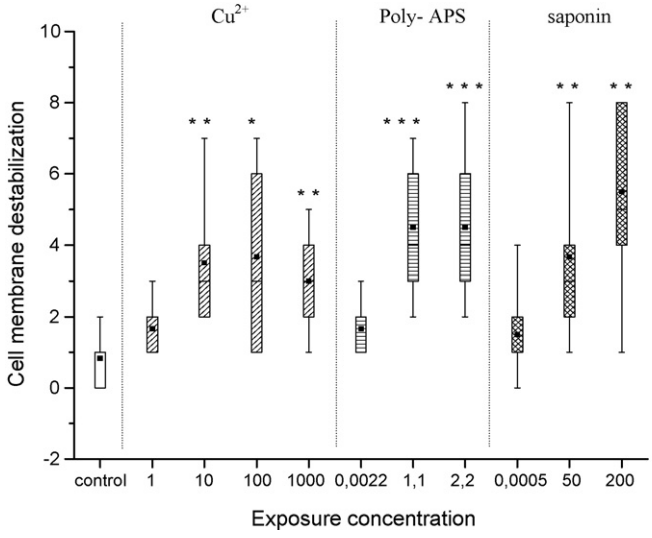

Fig. 3. Membrane integrity (nuclei stained with EB) of hepatopancreatic cells after oral application of different concentrations of Cu^{2+}, poly-APS and saponin. Statistical differences between control group and exposed groups are marked as different numbers of stars ($p < 0.05$ – *, $p < 0.01$ – **, $p < 0.001$ – ***).

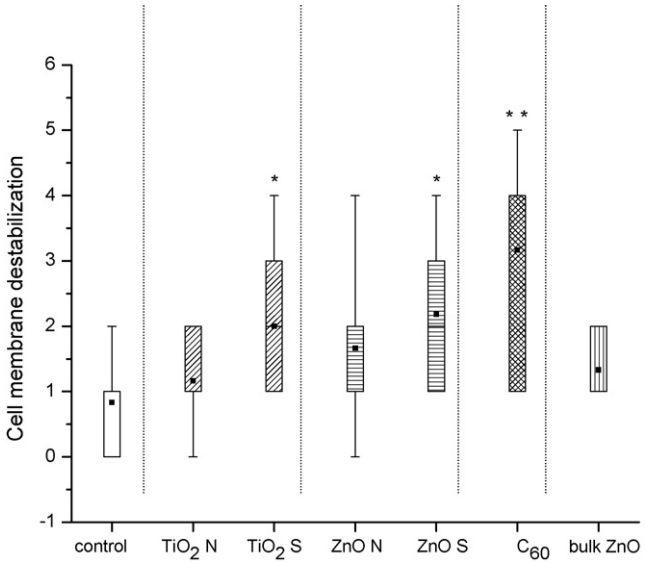

Fig. 4. Membrane integrity (nuclei stained with EB) of the hepatopancreatic cells after oral application of nanoparticles. Statistical differences between control group and exposed groups are marked as different numbers of stars ($p < 0.05$ – *, $p < 0.01$ – **).

ticle suspension dispersed in distilled water or in a physiological solution were compared. Differences in effects between distilled water suspension of nanoparticles and physiological solution suspension of nanoparticles on destabilization of cell membrane were not detected. In animals treated with non-sonicated TiO_2 or ZnO at concentrations of 1 mg/ml, the cell membrane permeabilities did not significantly differ from those in control experiments. Statistically significant differences compared to controls were observed upon oral application of sonicated TiO_2 and ZnO nanoparticles. The ability of C_{60} to destabilize the cell membrane *in vivo* was tested by oral application of sonicated suspensions at concentrations of 1 mg/ml in distilled water. In animals treated with sonicated C_{60} nanoparticles, the cell membrane destabilization was significant compared to the control (Fig. 4).

3.4. Oral application of bulk ZnO

The ability of bulk ZnO to destabilize the cell membrane *in vivo* was also tested by oral application of non-sonicated suspensions. Bulk ZnO did not affect cell membrane permeability.

4. Discussion

The AO/EB assay was modified for analysis of cell membrane permeability in a single-layer epithelium of digestive glands of a model test organism, terrestrial isopod *P. scaber* after *in vivo* exposure to particles or chemicals. This assay was employed to classify nanosized TiO_2, ZnO and C_{60} according to their membrane destabilization potential.

The AO/EB assay was validated using chemicals with known membrane destabilization effect. These were $Cu(NO_3)_2$ solution and two surfactant solutions which serve as positive controls. Acute oral application of Cu^{2+} resulted in a dose-dependent rise in cell membrane permeability. This may be due to increased lipid peroxidation caused by an excess of ROS [26]. Direct effect of Cu^{2+} on the cell membrane is also a possibility, but is less well understood. Acute oral application of different concentrations of saponin as well as acute oral application of poly-APS caused a concentration-dependent rise of the permeability of the cell membrane as well. This is explained by the pore forming mode of action of the surfactants [20]. These results prove that the method is appropriate for testing membrane destabilization potential of different agents. The application of AO/EB assay has been so far limited to *in vitro* cell studies. Modification of the AO/EB assay allowed application of the method to assess the biological potential of nanoparticles in organisms under realistic exposure scenario.

In the past years, a majority of nanotoxicity research has focused on cell culture systems. However, the data from these studies could be misleading and will require verification from animal experiments. Only the complex *in vivo* systems could reveal potential unique responses to interactions of the nanostructures with biological components inside the organism [27].

When the membrane destabilization potential of three different types of nanoparticles was compared, fullerenes were found to permeabilize cell membranes more efficiently than TiO_2 and ZnO. The AO/EB assay allows differentiation between sonicated and non-sonicated suspensions of nanoparticles. Oral application of sonicated nanoparticles resulted in the induction of cell membrane permeability but non-sonicated nanosized TiO_2 and nanosized ZnO did not cause destabilization of cell membranes. A possible explanation for this may lie in the fact, that in the process of sonication, smaller and thus potentially more biologically active agglomerates of nanoparticles are formed. A similar effect can be observed when comparing nanosized ZnO and bulk ZnO. As we expected, bulk ZnO had no destabilizing effect on membranes.

Different mechanisms of permeabilization of cell membrane by nanoparticles have been proposed by other authors [28]. Nanoparticles can induce oxidative stress for example [29,30] which in turn leads to lipid peroxidation and cell membrane permeabilization. Studies on juvenile largemouth bass brain cells confirmed that fullerenes do in fact induce oxidative stress [31]. A second possible mechanism of nanoparticle membrane destabilization involves oxidation of the lipid membranes, as a result of direct interaction between nanoparticles and lipids. For example, in the case of nanosized TiO_2, efficient destruction of bacteria has been ascribed to ultra-structural alterations of membranes, especially when irradiated with visible light [32]. In the case of nanosized ZnO, external generation of hydrogen peroxide has been considered to be one of the primary factors of antibacterial activity [33]. In the case of C_{60} [6], it has been suggested that induction of lipid peroxidation by C_{60} can result from direct physical contact with biological membranes.

5. Conclusions

The AO/EB assay is applicable for ranging chemicals and nanoparticles according to their cell membrane destabilization potential. When the same concentrations of nanosized TiO_2, ZnO and C_{60} were tested, C_{60} proved to be the most biologically potent. Differently pretreated particles differ in their biological activity. Sonicated nanoparticles are more biologically aggressive than non-sonicated nanoparticles. As expected, bulk material failed to cause any membrane destabilization. The advantages of this modified method are: *in vivo* exposure scenario, controlled administration via mouth and short duration of the test. Results are obtained within 1 h. In this system, biological effects of nanoparticles are tested under realistic conditions which include variety of alterations of nanoparticles inside the body before they interact with cells. The alterations of nanoparticles when they interact with biological fluids could not be imitated in *in vitro* cell culture tests.

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