Use of scanning electron microscopy to monitor nanofibre/cell interaction in digestive epithelial cells

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Conflict of Interest statement.

We declare that no conflict of interest is present.

ABSTRACT

We provide data obtained by scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS) on the interaction of ingested tungsten nanofibers with epithelial cells of the digestive tubes of a test organism *Porcellio scaber*. Conventional toxicity endpoints including feeding behaviour, weight loss and mortality were also measured in each investigated animal. No toxicity was detected in any of exposed animals after 14 days of feeding on tungsten nanofiber dosed food, but when nanofibers enter the digestive system they can react with epithelial cells of the digestive tubes, becoming physically inserted into the cells. In this way, nanofibers can injure the epithelial cells of digestive gland tubes when they are ingested with food. Our SEM data suggest that peristaltic forces may have an important role, not predicted by *in vitro* experiments, in the interactions of nanomaterials with digestive intestinal cells.

Keywords: nanofibers, Scanning electron microscopy; digestive gland epithelial cells; digestive system; *Porcellio scaber*

1. INTRODUCTION

Natural and artificial nanoparticles and nanofibers are present in our environment but data on their interactions with cells of living organisms and consequences of these interactions for the entire organism are sparse [1, 2].

Nanomaterials (NMs) are defined as materials that have structural features with at least one dimension of <100 nm, and include nanofilms and nanocoatings (<100 nm) in one dimension, nanotubes and nanowires (<100 nm) in two dimensions and NMs (<100 nm) in three dimensions [3]. Tungsten oxides (WO_3, WO_2, and WO_x) have been considered for use in many important applications including optical devices, gas sensors, electrochromic windows, and photocatalysts [4]. Synthesis of tungsten oxides can be accompanied by release of fiber-like nanoparticles and this raises safety concerns reminiscent of those associated with asbestos fibers [5, 6]. Large scale production of WO_x nanofibers (nano-WO_x) can also result in environmental pollution. Because they can cause free radical damage *in vitro* WO_x nanofibers, either as whiskers or needles, are recognised as being more biologically potent than non-fibrous WO_x [7]. Particles of tungsten carbide (WC) that can cause pneumoconiosis are also well known [8].

Consequently, development of a hazard profile is a critical step in characterizing the potential safety of nanofibers, and the associated health and environmental hazards. The most important routes of exposure are *via* respiratory and digestive systems but although there are some *in vitro* studies on appropriate cell models, *in vivo* data are scarce.

In this study we have examined the effects of ingested tungsten nanofibers *in vivo* on a model invertebrate terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea), an organism of choice for testing effects of different ingested substances. Their digestive glands (hepatopanceas) resemble liver and pancreas of vertebrates. Hepatopancreatic cells are directly exposed to substances in partly digested food, and filtered and transported from stomach into the lumen

of the hepatopancreas. The digestive gland epithelium is subject to complex physical forces *in vivo* engendered by contact with luminal contents and pressure from peristalsis.

The objective of this work was study by SEM and EDS of the epithelial surfaces of control potential physical interactions between ingested nanofibers and cells *in vivo*. Such morphological studies have been reported on cell cultures [9, 10], but in our *in vivo* study some crucial parameters such as peristalsis, that govern digestion are included. We have combined data on interactions between nanofibers and digestive gland cells with data on conventional toxic responses of these organisms and we discuss the mechanical interactions between ingested nanofibers and cells and potential toxic outcome.

2. MATERIALS AND METHODS

2.1 Experimental animals

We have used the woodlouse terrestrial isopod *Porcellio scaber* to test effects of nanofibers on the digestive gland tube *in vivo* (Figure 1a). Terrestrial isopods, including *P. scaber*, have become organisms of choice in (eco) toxicology, (eco) physiology and recently nanotoxicity studies due to a lot of physiological, stress and toxicological biomarkers that could be controlled under in the laboratory where the exposure dose as well as exposure concentration to selected substance could be assessed [11, 12, 13, 14, 15, 16, 17].

Individual *P. scaber* were collected in three different locations near Ljubljana. The animals were kept in a terrarium (20/35/20 cm) for acclimatization for a period of three months. The terrarium was filled with a 2-5 cm layer of moistened sand and soil and a thick layer of partly decomposed hazelnut (*Corylus avellana)* tree leaves that had been collected in uncontaminated woodland and dried at room temperature. The substratum in the terrarium was heated to 80 °C for several hours to destroy predators (spiders) before the introduction of

the isopods. The culture was kept at controlled room temperature (21 ±1°C), a 16:8-h light-dark photoperiod, and high humidity.

2.2 Preparation and characterisation of nanofibers

WO_x nanofibers that were used in this study were synthesized by a chemical transport reaction [18] from tungsten powder (99.9%) and WO_3 powder (99.9%) in the stoichiometric ratio of $WO_{2.86}$. Iodine (99.8%) in a volume fraction of 3.2 mg/cm^3 was added as a transport agent. The material was transported from the source (hot zone at 1123 K) to the growth zone (1009 K), with a 5.7 K/cm temperature gradient. The nanofibers were studied using transmission electron microscopy (Figure 1c) (200 keV Jeol 2010F), and scanning electron microscopy (Figure 1b) (FE-SEM, Supra 35 VP, Carl Zeiss).

2.3 Experimental set-up

The nano-WO_x material was suspended in distilled water before application, to obtain the final concentration of 1000 µg nano-WO_x/ml. To diminish aggregation of nanofibers in distilled H_2O the suspension was sonicated in an ultrasonic bath for 1h and mixed using a vortex mixer just before applying on the leaves. Before the application of the dispersion of tungsten oxides nanofibers, the leaves were kept in humid Petri dishes to facilitate the absorption of the dispersion of nanofibers. 1000 µl of the dispersion per 100 mg of leaf were applied to the lower leaf surfaces. In the control group, the leaves were treated with distilled water. The suspension of nanofibers or the distilled water was brushed onto the abaxial leaf surface to give final nominal concentrations of nanoparticles on the leaves of 1000 µg nano-WO_x per gram (dry wt) of leaf and allowed to dry.

Subsequently, the leaves were dried for a further 24 h at room temperature. Two experiments were conducted and altogether 13 animals were exposed to nano-WO_x; 8 were used as controls and were exposed to untreated leaves.

After an exposure period of two weeks, the animals and remnants of food were weighed to calculate the weight change and feeding rate. Before weighting, the food was dried at room temperature for 48h.

2.4 SEM/EDX investigation

Before dissection, the animals were anesthetised by cooling, decapitated, and the digestive glands (hepatopancreas) were isolated and prepared for SEM using our standard protocol for biological samples. The hepatopancreas was transferred with tweezers to a fixative containing 2.5% glutaraldehyde, 0.4% paraformaldehyde and 0.1M sodium phosphate buffer (pH 7.2). After primary aldehyde fixing, the digestive glands were put in 1% osmium tetroxide and stained with TOTO (thiocarbohydrazide/ osmiumtetroxide/ thiocarbohydrazide/ osmiumtetroxide) conductive stain, a method described previously by Lešer at al. [19]. The fixed hepatopancreas glands were dehydrated in absolute alcohol and dried with hexamethyldisilizane (HMDS) and the dry samples were mounted on aluminium holders and coated with a 5 – 10 nm layer of gold/palladium alloy. The coating of samples was accomplished with a precision etching coating system (Gatan Model 682). Digestive tubes from the control group and exposed groups were investigated with the SEM JSM-6500F scanning electron microscope with a field emission electron source and the presence of tungsten was detected by energy dispersive x-ray spectroscopy (EDS).

3. RESULTS

SEM revealed that in 9 animals out of 13 animals exposed to nano-WO_x, the tungsten nanofibers were embedded into cells of the digestive gland epithelium (Figure 2 a-d). The fibers were observed always to be thrust into lateral parts of cells, which is possibly a consequence of forces generated by peristaltic movement of glands (Figure 3 a, b). When fibers are situated in the spaces between the cells (Figure 3 c, d), they can be pushed into the cells after contraction of circular muscles. When radial muscles of the tube are contracted, the cells are pressed together with lateral parts. After relaxation of these muscles, the lateral parts of digestive gland cells move apart again.

Fibers are observed to be embedded in <10% of the cells but due to dense bacterial colonisation and in some cases, other organic material covering the cell surfaces (Figure 4a, b), the fibers may not be observed. Quantitative evaluation of the amount of affected cells and the most affected region of gland tube is currently being carried out.

The presence of nano-WO_x in digestive gland cells (Figure 5a) was confirmed by EDS elemental analyses (Figure 5b). As expected, the surface of nanofibers was covered by organic material from the gland lumen. In the animals exposed to WO_x nanofibers for 14 days, no alterations in feeding rate, weight and mortality were detected. Since these conventional toxicity parameters were not affected, we conclude that this exposure dose was not toxic.

4. DISCUSSION

In this study we present experimental evidence of physical interactions *in vivo* between tungsten nanofibers and digestive gland epithelium. We show that after 14 days feeding on food containing 1000 μg nano-WOx per gram (dry wt) food, nanofibers are found to be inserted into digestive gland cells but no more than 10% of the animals are affected. In

addition, no toxic responses, as measured by conventional toxicity markers like feeding rate, weight change or mortality were observed. These data confirm that cellular effects of nano-WO_x are not necessary propagated upwards to cause toxicity.

In some studies evidence has been provided that cellular effects caused by nano-TiO_2 did not result in cell death and it was pointed out that although nanoparticles are not shown to be toxic by conventional toxicity and cytotoxicity biomarkers, nanoparticles could lead to more subtle, non-lethal changes in cells [10]. Some authors also demonstrated that highly purified carbon nanotubes possess no evident short-term toxicity and can be considered biocompatible with cardiomyocytes in culture, and they suggested that while the long-term negative effects, which were observed after reseeding, were the result of physical rather than chemical interactions [20].

There are consistent literature reports that size of nanofibers and nanotubes has a crucial role in their biological potential. Schinwald and Donaldson [21] have recently reported length-dependent effects of a range of fibers including asbestos, carbon nanotubes, silver nanowires (AgNW) and nickel nanowires (NiNW) at the peritoneal and pleural mesothelial surfaces. However, there are no reports on the role of mechanical forces generated by muscular contraction on physical interactions between cells and nanofiber like particles. In this study we provide evidence that muscle contraction during peristalsis may contribute to fiber insertion in cells. Since we found fibers embedded into the cells only at their lateral parts we assume that physical interactions are also an important factor.

Our results suggest fact that nanomaterials could lead to conditions in which the lining of the gastrointestinal tract becomes affected with no clear cause and without immediate toxic response. One of the most important portals of entry for nanomaterials is the gastrointestinal tract (GI) [22]. The fundamental concepts and processes of absorption and food passage through digestive system in the invertebrate model we have used could also be applied to

humans and other mammalian and some invertebrate species. Our results point to the fact that nanomaterials could lead to conditions where the lining of the gastrointestinal tract becomes affected, but with no clear cause and without immediate toxic response.

Since the early days of the REACH proposals [23], it has been generally agreed that the number of animals used to gain toxicity information on chemicals should be kept to an absolute minimum. Because of this, most recent studies on biological effects of nanoparticles are conducted *in vitro*. *In vitro* studies are satisfactory models to study cellular responses to selected agents, but are less useful when organism level responses are sought. For example, the physical insertion of nanofibers into cells has probably been overlooked in *in vitro* studies. We suggest that when biological effects and toxicological potential of nanofibers and nanotubes are to be examined, invertebrate animal substitutes are a better choice than *in vitro* cell cultures.

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Figure captions:

Figure 1. Model organism and nanofibers used in the study. a) *Porcellio scaber* with digestive glands shown. b) Scanning electron micrograph of WO_x nanofibers. c) Transmission electron micrograph of WO_x nanofibers.

Figure 2. Interaction of nanofibers with epithelial cells. NF- WO_x nanofibers, C- epithelial cells a) Part of nanofibers embedded into the cell membrane. b) Broken parts of nanofibers. c) Nanofiber who is inserted deeply into the cell. d) Nanofibers damaged by peristalsis.

Figure 3. Digestive glands of *Porcellio scaber* investigated with scanning electron microscopy. a) Digestive gland tube with nerve (N) on surface. b) Radial muscle (M) of digestive gland tube. c) Cells (C) of digestive gland tissue. c) The spaces (Sp) between the digestive gland cells.

Figure 4. Scanning electron micrography of digestive gland cells. a) Surface of digestive gland cell with dense bacterial colonisation. b) Digestive gland cells surface covered with organic material.

Figure 5. Energy dispersive x-ray spectroscopy (EDS) spectra of WO_x nanofibers trusted in cell. a) Position where EDS spectra was taken (fiber like structure trusted in digestive gland cell) b) EDS spectra confirming presence of tungsten trusted in cell in Figure 5a.

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