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# Micro-PIXE study of Ag in digestive glands of a nano-Ag fed arthropod (*Porcellio scaber*, *Isopoda*, Crustacea)

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ABSTRACT

Micro-proton induced X-ray emission (micro-PIXE) method was applied to study the micro-localization of silver (Ag) in digestive glands of a terrestrial arthropod (*Porcellio scaber*) after feeding on silver nanoparticles (nano-Ag) dosed food. The aim of our work was to assess whether feeding on nano-Ag results in the assimilation of silver (Ag) in digestive gland cells. To study micro-localization and elemental distribution of Ag, the animals were fed on food dosed with nanoparticles for 14 days under controlled laboratory conditions. At the end of the feeding exposure, the animals were dissected and digestive glands prepared for micro-PIXE analyses and TEM investigation. The results obtained by micro-PIXE documented high amounts of Ag inside S-cells of the digestive gland epithelium; however, TEM investigation did not show particle aggregates inside digestive gland cells. Also no adverse effect on feeding behavior was recorded what is a measure of toxic effects. We explain the presence of Ag inside the cells as a result of the assimilation of dissoluted Ag ions from ingested nano-Ag particles. Assimilation of excessive amounts of ingested metal ions in S-cells is a well known metal detoxification mechanism in isopods. We discuss the advantages of using micro-PIXE for the micro-localization of elements in biological tissue in studies of interactions between nanoparticles and biological systems.



## 1. Introduction

Among the 580 consumer products containing known nanomaterials, Ag-based nanoparticles are the most commonly mentioned in product descriptions [1]. Known for their anti-microbial activity these were developed in order to improve human health [2] and they are found in disinfectants, deodorants, anti-microbial sprays and powders, bedding, machine washers, humidifiers, water purification and air filters, toothpastes, shampoos and rinses, re-usable bottle nursing nipples, and in multiple fabrics, kitchen utensils and toys [3]. With increased application of nano-Ag products inevitably comes the possibility of adverse effects on humans and on the environment [4].

There are already several reports on toxic effects of nano-Ag *in vitro* [5,3,6–8] and *in vivo* [9–12] systems. Fewer data are available on the absorption of nano-Ag by organisms themselves. Entry of nano-Ag into organisms may occur by inhalation, or oral or dermal routes, however, there is little information on the subsequent distribution of the nano-Ag and its possible accumulation in specific tissues [13,14,1].

Bioaccumulation of metals can be studied by different techniques which provide data on the distribution and chemical state of elements in biological systems at the cellular level [15–17]. Imaging techniques based on X-ray fluorescence currently rank among the most sensitive modalities for detection of elements in biological samples with submicrometer resolution [17]. These are microprobe methods which use electron beams, proton beams or X-ray beams and rely on the excitation of the atom's core-shell electrons, which subsequently relax, emitting photons. Among these techniques, the energy dispersive X-ray analysis (EDX) in a transmission electron microscope provides the highest lateral resolution, but only a moderate chemical sensitivity [16]. It requires specimen analysis performed *in vacuo*, preparation of thin sections of a specimen, and a conductive sample surface. An alternative technique, particle induced X-ray emission (PIXE) is gaining importance in the analysis of elemental distribution and concentration in biological samples at the tissue level and delivers lateral resolution in the micron range [18].

In the present paper, we report on the bioaccumulation of Ag in the digestive gland epithelium (hepatopancreas) of a terrestrial isopod *Porcellio scaber* fed on nano-Ag dosed food. Digestive glands

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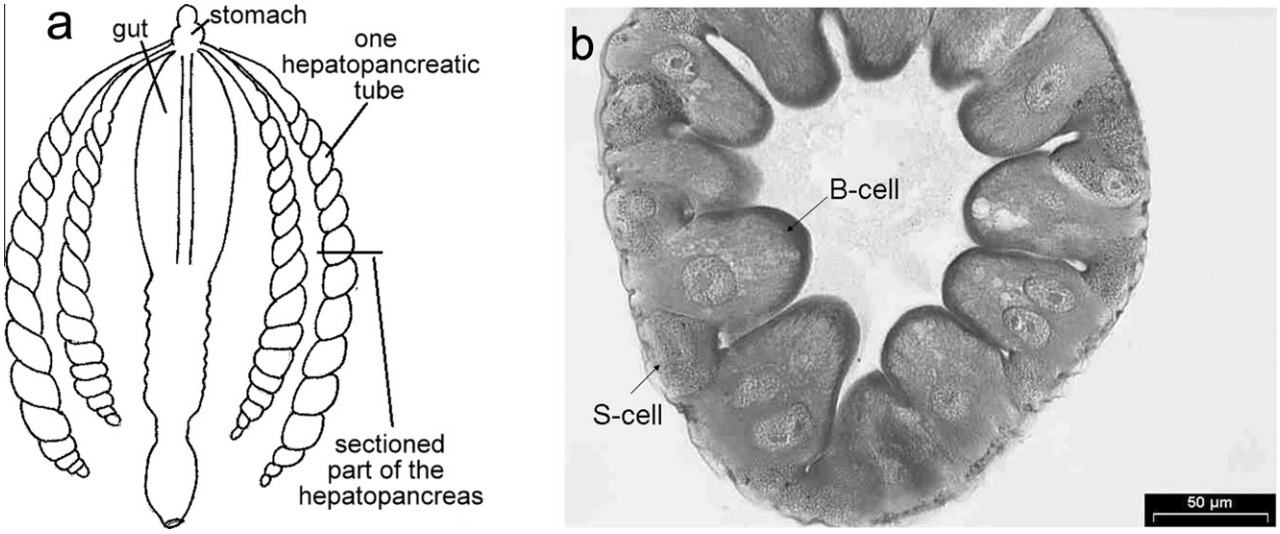


**Fig. 1.** (a) Schematic diagram of the digestive system of the *Porcellio scaber*, (b) optical micrograph of a cross-section of a digestive gland tube, consisting of dome-shaped B-cells and wedge-shaped or cylindrical S-cells.

of isopods are composed of four blind-ending tubes within the body cavity. The hepatopancreatic epithelium contains two cell types, large B-cells and wedge-shaped S-cells, both of which occur alternately in cross-sections [19] (Fig. 1). The B-cells are secretory and absorptive; they usually contain glycogen and many lipid droplets [19,20]. The S-cells contain Ca and urate and accumulate large amounts of metals [19–21]. Spherical granules in the S-cells contain Cu, S and Ca. In isopods from contaminated sites, these so-called 'copper' granules also contain Zn, Cd and Pb. Iron granules, which also contain Zn and Pb have been observed in the B-cells in tissues of animals from contaminated sites [20].

In our study, which is focused on the elemental distribution in tissue, micro-PIXE appeared to be the analytical method of choice. In order to visualize potential internalization of nanoparticles and intracellular formation of particle aggregates, we performed ultrastructural studies of the same tissues.

The aim of this work was to determine whether ingestion of nano-Ag results in the assimilation of Ag in digestive gland cells and the formation of nanoparticle aggregates inside cells. We discuss the broader applicability of this approach in studies of interactions between nanoparticles and biological systems.

## 2. Experimental

### 2.1. Nanoparticles

Nano-Ag particles (Sigma-Aldrich, 99.5% purity, particle size <100 nm; surface area, 5.0 $m^2/g$) were suspended in distilled water with a vortex (20 s, 18g). The suspension was prepared immediately prior to application onto leaves.

### 2.2. Experimental design

Terrestrial isopods (*Porcellio scaber*) were collected under litter in an uncontaminated location in the vicinity of Celje, Slovenia. In the laboratory, the animals were kept in a terrarium filled with a 2–5 cm layer of moistened soil and a layer of partly decomposed leaves of a hazelnut tree (*Corylus avellana*). The culture was kept at controlled room temperature (21 ± 1 °C), at 16:8 h light: dark photoperiod, and high humidity.

The adults of *P. scaber* of both sexes and all molt stages with body weights ranging from 30 to 70 mg, were used in the experiment. Each animal was placed individually in a Petri-dish and pieces of nano-Ag dosed leaf (~100 mg) or uncontaminated control leaf were added. The humidity in the Petri-dish was maintained by regularly spraying tap water on the internal side of the lids of the Petri-dishes. During the course of experiment, the Petri-dishes were placed in a large container and kept under controlled conditions in terms of humidity (⩾80%), temperature (21 ± 1 °C) and light regime (16:8-h light: dark photoperiod). The experiment period was 14 days.

Nanoparticle dosed food was prepared by applying the suspension of nanoparticles to the surface of dry hazelnut tree leaves. The leaves were collected in uncontaminated woodland and dried at room temperature. Dried leaves were cut into pieces of similar surface area and then weighed. Pieces of approximately 100 mg were used in the experiments. Just prior to application, five different suspensions of nano-Ag particles were prepared and evenly spread over the lower leaf surfaces with a brush to obtain five final concentrations of Ag on leafs. The exposure concentrations were selected on the basis of our preliminary studies and were 0.1, 10, 100, 1000 and 5000 μg/g nano-Ag. Distilled water was applied on the leaves in the control group. For the experiments, a group of animals consisted of 10–14 individuals.

At the end of the experiment, the animals were anaesthetized at low temperature and then decapitated. The digestive glands were isolated and prepared for micro-PIXE analyses and ultrastructural study. For this type of analysis up to four animals were sacrificed. The tissue of the same animal was prepared for both micro-PIXE and TEM investigations.

### 2.3. Assessment of feeding rates of animals

Fecal pellets of each animal were collected after 5 and 10 days of exposure, and counted and weighed after drying at room temperature for 24 h. After 14 days of exposure, the remaining leaves and fecal pellets were dried at room temperature for 24 h and weighed. The animals were also weighed at the end of the experiment. The feeding rate of each animal was expressed as the mass of consumed leaf per animal wet weight per day.

### 2.4. Micro-PIXE analysis

For micro-PIXE analysis digestive glands were rapidly frozen in liquid $N_2$ using tissue freezing medium (Jung Tissue Freezing Medium, Leica). Samples were sectioned with a Leica CM3050

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cryotome (Leica, Bensheim, Germany) with the temperature of the microtome head and chamber between −25 and −20 °C and a section thickness of 60 μm. The sections were placed in pre-cooled Al holders and transferred to an alpha 2–4 Christ freeze dryer using a cryo-transfer-assembly cooled with liquid nitrogen, and then freeze–dried at −30 °C and a pressure of 0.4 mbar for 24 h. Dry sections of animals were mounted on the Al sample holder between two thin layers of Pioloform foil [18,22,24,25].

For the detection of the X-ray energies ranging from 1 up to 25 keV, a pair of X-ray detectors was used. A high-purity germanium X-ray detector (active area 95 $mm^2$, 25 μm thick beryllium window, 100 μm thick polyimide absorber) positioned at 135° with respect to the beam direction was used for the energy range of 4–25 keV. Low energy X-rays in the range of 0.8–4 keV were detected by a Si(Li) detector (active area 10 $mm^2$) positioned at 125° with respect to the beam direction. The proton dose was determined by a rotating beam chopper. Measurement of micro-PIXE and data evaluation for biological samples of intermediate thickness at the micro-PIXE setup at the Jožef Stefan Institute in Ljubljana has been described in detail previously [18,23,24]. Micro-PIXE scans indicate the positions of the S-cells in the digestive glands in the samples with high contrast in the copper and zinc maps (Fig. 2), as they accumulate large quantities of Cu and Zn [21].

### 2.5. Ultrastructural studies

For ultrastructural observation animals were dissected, digestive glands isolated, transferred to 2.5% glutaraldehyde in 0.1 M phosphate buffer, postfixed with 1% $OsO_4$ and embedded in plastic (Agar 100 resin kit, Agar scientific, Stansted, UK). We omitted the conventional contrasting with heavy metals in order to detect possible nano-Ag aggregates inside digestive gland cells of nano-Ag fed animals.

Ultrathin sections were cut with a diamond knife on an ultramicrotome (Reichert Ultracut S, Liechtenstein) and examined with a Philips CM 100 TEM (Department of Biology, University of Ljubljana). Images were recorded with a Bioscan 792 camera (Gatan, UK).

## 3. Results

### 3.1. Nanoparticles

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) revealed spherically shaped particles between 30 and 200 nm in diameter. The majority of the particles were found as aggregates (Fig. 2).

### 3.2. In vivo effect of nanoparticles

For this study we measured two parameters (biomarkers) which are commonly used to assess the adverse effect of chemicals *in vivo* with the terrestrial isopod *P. scaber*. These are feeding behavior and weight change. Neither of these two parameters showed any adverse effect due to the presence of nano-Ag in the food. There were no changes in feeding rate of *P. scaber* fed on food dosed with up to 5000 μg/g nano-Ag for 14 days (Fig. 3) and there were also no changes in animal's weights during the experiment. We concluded that the concentrations tested were no observed effect concentrations (NOEC).

### 3.3. Micro-PIXE analyses

Micro-PIXE analyses show the presence of Ag predominantly in the digestive gland epithelium of animals fed on nano-Ag dosed food. Silver K X-rays are detected (Fig. 4) with a statistics, which

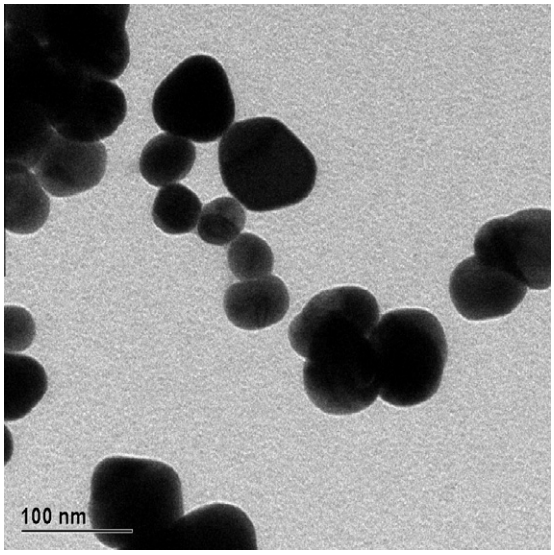


**Fig. 2.** Transmission electron micrograph of nano-Ag.

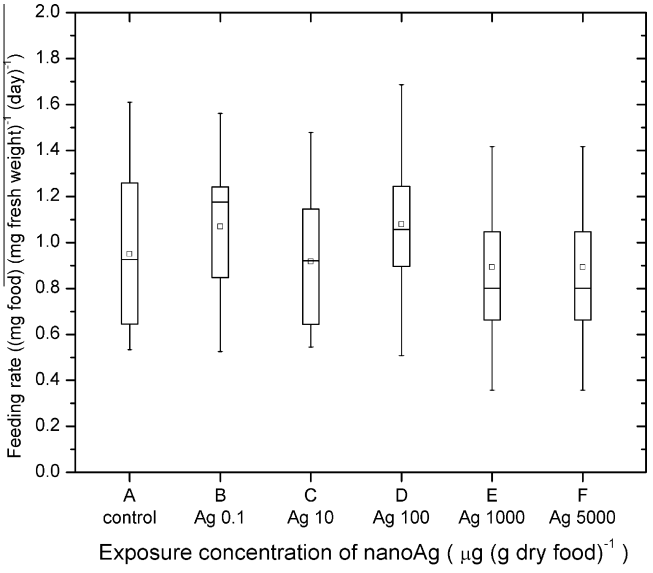


**Fig. 3.** The feeding rate of animals fed with nano-Ag dosed food. Symbols on the box plot represent maximum and minimum value (whiskers) and mean value (square).

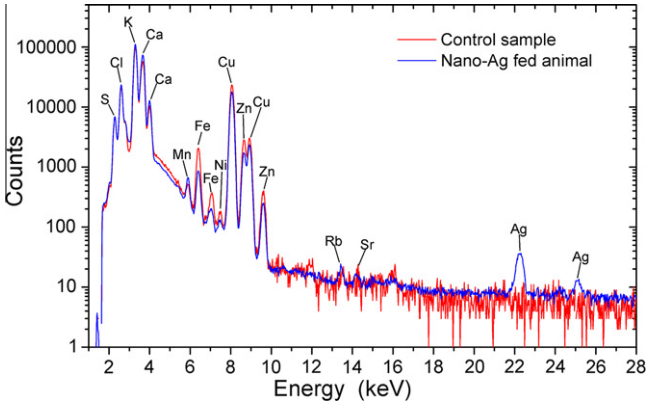


**Fig. 4.** Micro-PIXE spectrum measured by germanium X-ray detector on the section of digestive gland tissue: control sample and nano-Ag fed animal.

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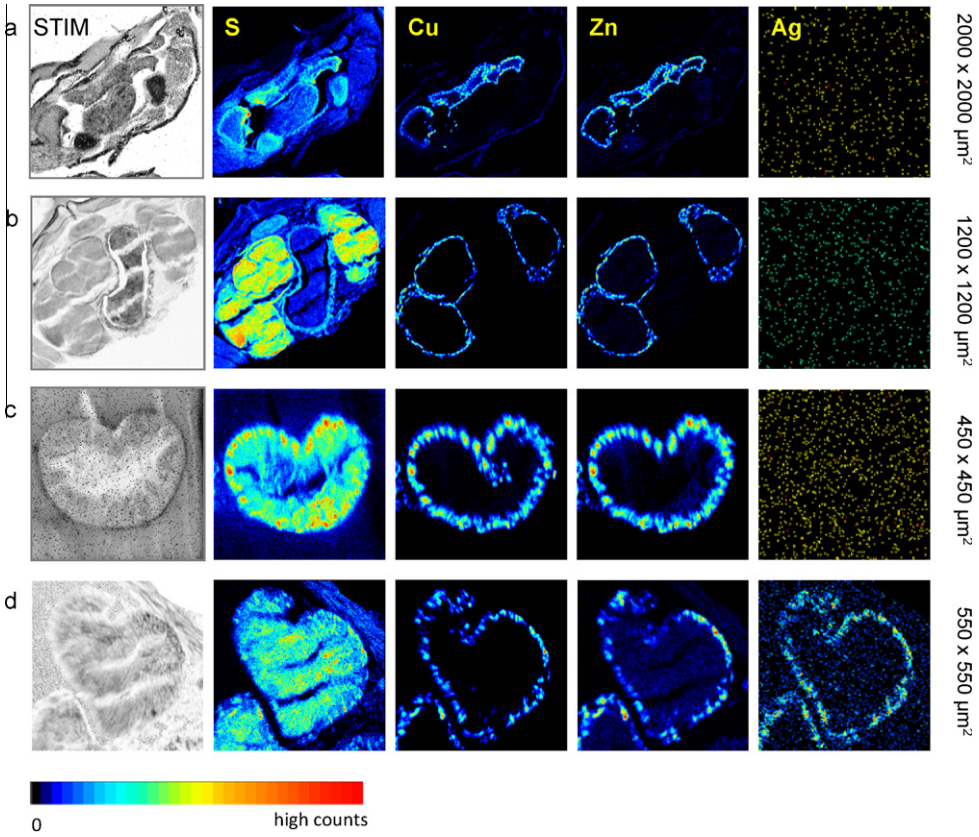


**Fig. 5.** Scanning Transmission Ion Microscopy (STIM) images and micro-PIXE qualitative elemental maps of S, Cu, Zn and Ag of *Porcellio scaber*: (a) section along the body axis of control animal; (b) section perpendiculary to the body axis of a control animal; (c) cross-section of a digestive gland of a control animal; (d) cross-section of a digestive glands of a nano-Ag fed animal from a laboratory experiment.

allows a reasonable two-dimensional mapping (Fig. 5d). In control animals, Ag was not found inside digestive gland cells (Fig. 5a–c). Since the spatial distribution of Ag in digestive gland epithelium corresponds to that of Cu and Zn and co-localization of Ag and Cu is evident, we assume that Ag is also stored in S cells.

### 3.4. Ultrastructural studies

There were no differences between controls and nanoparticle fed animals in the presence of electron dense aggregates in digestive gland cells (Fig. 6a and b) investigated by TEM. In control animals and also in nano-Ag fed animals we observed electron dense material. We did not found any aggregates, which would indicate nano-Ag. We examined the same region of the epithelium where Ag was observed by the micro-PIXE method. As expected, metal storage granules were detected in S-cells. These corresponded to the Cu-granules described by many authors. No significant differences in the appearances of these Cu-granules were observed when control animals and those fed on nano-Ag dosed food were compared (Fig. 6c).

## 4. Discussion

Evidence is provided that feeding on nano-Ag dosed food results in assimilation of Ag in the digestive gland epithelium cells of a model invertebrate animal. Ultrastructurally we could not detect the formation of electron dense aggregates corresponding to internalized nanoparticles and no effects on feeding behavior and weight of animals fed for 14 days on up to 5000 µg/g nano-Ag in the food were observed.

Elemental distribution in digestive gland cells determined by the micro-PIXE method showed that Ag, Cu and also Zn maps overlap. It appears that majority of the Ag is in small cells of the digestive glands which are known to accumulate metals [26]. Reports in the literature indicate that Ag accumulation results from free Ag ions that are bioavailable [1]. Ion release rates from nano-Ag increase with temperature in the range 0–37 °C, and decrease with increasing pH or the addition of humic or fulvic acids. Silver nanoparticle surfaces can adsorb $Ag^+$, so even simple colloids contain three forms of Ag: $Ag^0$ solids, free $Ag^+$ or its complexes, and surface-adsorbed $Ag^+$. Both thermodynamic analysis and kinetic measurements indicate that $Ag^0$ nanoparticles will not persist in realistic environmental compartments containing dissolved oxygen [27]. In isopods, there are two types of granules which accumulate metal ions. These are Type B and Type C granules. In Type B Cd, Cu, Hg and Ag were reported to accumulate. In Type C granules Fe is usually found. We did not find a co-existence of Ag and Fe, but a parallel existence of Cu and Ag. This is in accordance to literature data [20]. The observation that Ag, but not Fe is found in the same micro-locations as Cu and Zn suggests that ionic forms of Ag, but not entire particles of Ag were accumulated. The appearance of Cu-granules in S-cells of digestive gland epithelium indicates that Ag ions and not Ag nanoparticles are stored in the cells. Cu-granules are composed of homogeneous electron dense material and are up to 1 µm in size. When nanoparticles are internalized, they preserved the nanoparticle's size and original shape. Within cells they are commonly in membrane bound vesicles [28,29].

Many authors also report cellular internalization of nanoparticles, which are detected as membrane bound micron-sized clusters everywhere in the cytoplasm [28,29]. We observed membrane

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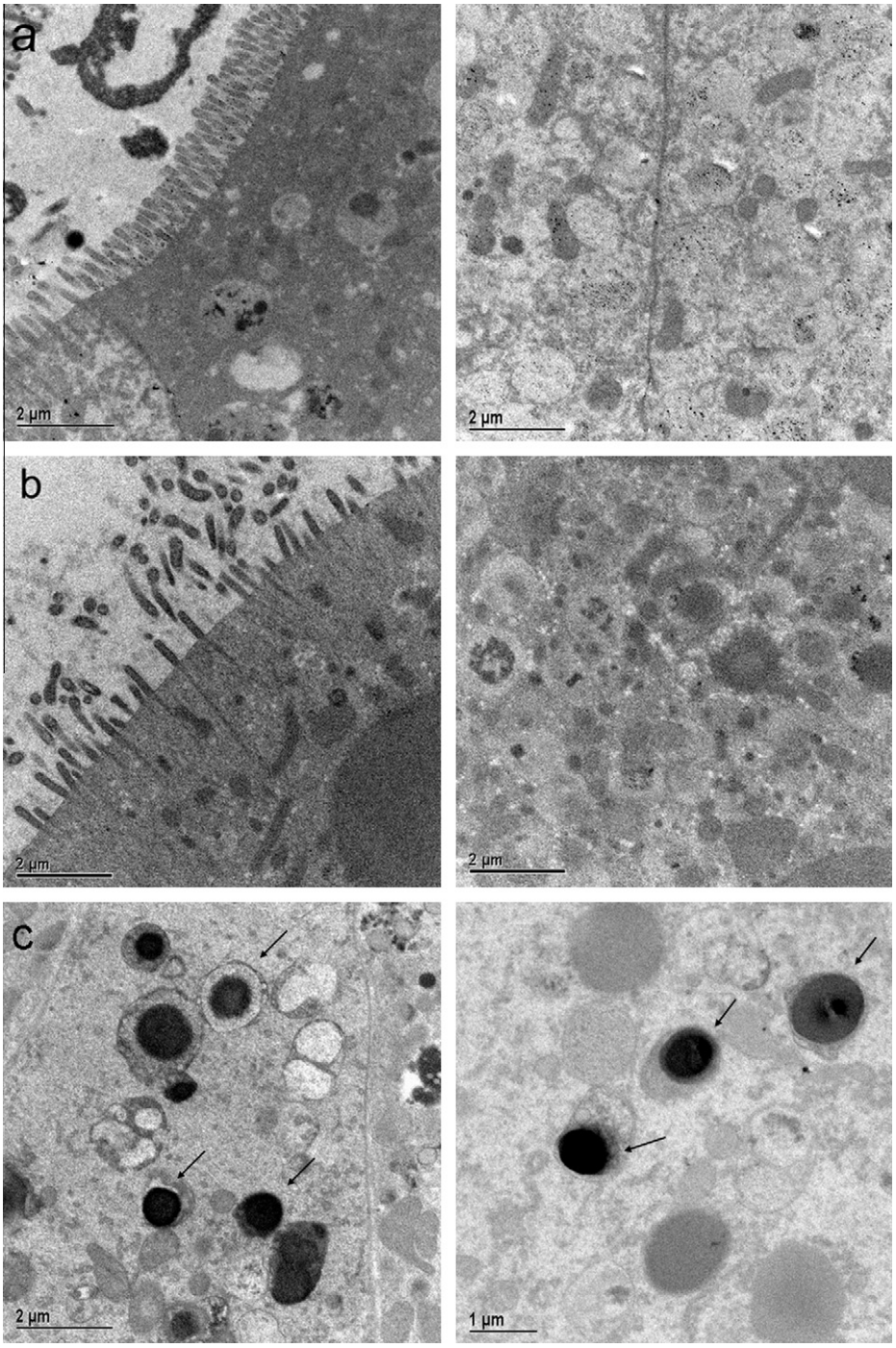


**Fig. 6.** Transmission electron micrographs of digestive gland epithelium: (a) control group (on the left side B-cell and on the right side S-cell with electron dense material); (b) nano-Ag fed animals *Porcellio scaber* from a laboratory experiment (B-cell and S-cell with electron dense material); (c) metal granules in S-cell (on the left is control group and on the right is nano-Ag fed animals *Porcellio scaber* from a laboratory experiment). Arrows indicate metal granules.

bound electron dense clusters in the cytoplasm of both cell types, but there were no differences between control and nano-Ag fed animals in the presence of these clusters. Therefore, it seems likely that the clusters are not related to the consumption of nano-Ag.

The inability to detect electron dense aggregates in digestive gland cells of nano-Ag fed animals does not exclude the presence of nanoparticles inside the cells, but other imaging modalities are necessary to gain this information. Synchrotron X-ray fluorescence microscopy (SXRF) is a technique which offers simultaneous analysis of trace element sensitivity and submicron spatial resolution combined with the ability to provide information regarding the oxidation state and degree of coordination of metals [30]. Research currently in progress is aimed at the determination of the oxidation state of elements detected inside cells and whether dissolved ions or nanoparticles, or both penetrated the cells.

In the study presented here, we used a terrestrial isopod to assess the toxic potential of substances added to food [31]. The results show that exposure concentrations up to 5000 μg/g nano-Ag in the food did not affect the feeding behavior and weight of the animals and are therefore considered to be non-toxic. The presence of Ag in digestive gland cells may be a result of a successful sequestration mechanism in which excessive amounts of ingested

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metals or metal ions are stored in metal granules predominantly in S-cell. This mechanism is viewed as a detoxification mechanism which helps the organism to cope with high amounts of metals in the environment. On the other hand, organisms which are able to accumulate bioavailable metals could serve as a biological system with which it would be possible to assess bioavailable metal fractions in the substances added to food [32] and the dissolution rate of ions from nanoparticles.

## 5. Conclusions

Our results show that the micro-PIXE method with its high sensitivity and good lateral resolution is a method of choice in studies where elemental distribution at organism/tissue levels is studied. Among the outstanding advantages of this method is chemical-free sample preparation. The micro-PIXE analysis, in combination with other methods could be of great benefit for studies of interactions between nanoparticles and biological systems.

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