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Effect of ingested tungsten oxide (WO\textsubscript{X}) nanofibers on digestive gland tissue:

Synchrotron-based Fourier transform infrared (FTIR) microspectroscopy

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Abstract

Tungsten nanofibers are recognised as biologically potent. We study deviations in molecular composition between normal and digestive gland tissue of WO$_x$ nanofibers (nano-WO$_x$) fed animals and reveal mechanism of nano-WO$_x$ effect on digestive gland cells of a model organism in vivo. Fourier transform infrared (FTIR) spectroscopy was performed on digestive gland epithelium of animals fed with WO$_x$ nanofibers. The FTIR analyses were supplemented by toxicity and cytotoxicity analyses as well as scanning electron microscopy (SEM) of the surface of the epithelium. The difference in the spectra of the WO$_x$-treated and control cells showed up in the central region of the cells and are related to a changed protein to lipid ratio, lipid peroxidation and structural changes of nucleic acids. The conventional toxicity parameters failed to show toxic effects of nano-WO$_x$, whereas the cytotoxicity biomarkers and SEM investigation of digestive gland epithelium indicate sporadic effects of nanofibers. The FTIR results are in agreement with toxicological and cytological measurements, which indicate that ingestion of nano-WO$_x$ does not affect severely the cell membrane stability and feeding behaviour. However, we explain changes observed by FTIR as protection of cells to unfavourable conditions and indication of non-homeostatic state, which can lead to toxic effects.
Introduction

Tungsten oxides (WO$_3$, WO$_2$, and WO$_x$), which have attractive semiconductor properties, have been considered for many important applications including optical devices, gas sensors, electrochromic windows, and photocatalysts.$^1$ Synthesis of tungsten oxides however can be accompanied by release of fiber-like nanoparticles which raises safety concerns reminiscent of those associated with asbestos fibers, which were found to be highly toxic inducing irreversible health problems.$^2$

Exposures to tungsten and its compounds in occupational environments include those during production of tungsten metal from the ore and fabrication of tungsten carbide powders in the tungsten refining and manufacturing industry (Agency for Toxic Substances and Disease Registry, 2005). WO$_x$ nanofibers, whiskers or needles are recognised as being more biologically potent than non-fibrous WO$_x$ due to their ability to produce free radical damage in vitro.$^3$ Tungsten carbide particles (WCs) that can cause pneumoconiosis$^4$ are also well known.

Detection of biological effects can be gained from comparisons of healthy and abnormal tissue, what can be carried out by a variety of physical, biological and biochemical methods. The selection of methods is based on the expected alteration but, when it is necessary to shed more light on molecular and functional changes, methods which can monitor a broad range of structural or functional alterations are required. Among these, Fourier Transform InfraRed (FTIR) microspectroscopy which uses IR radiation to detect deviations in molecular composition between normal and abnormal tissue is very promising.$^5$ This technique is based on absorption of infrared light by the vibrational transitions in covalent bonds and intensities provide quantitative information, while frequencies give qualitative information about the nature of these bonds, their structure, and their molecular
environment. FTIR microspectroscopy is a label-free, non-destructive and objective tool for discriminating between normal tissues and any alteration. In complex systems such as cells, the main spectral features arise from N-C=O, N–H, C=O, C–H and P=O bonds in proteins, lipids, and nucleic acids. The infrared spectrum of cells reflects all these contributions and provides information on the concentration, organization and structure of the most fundamental macromolecules.  

Interactions between cells and nanoparticles lead to alteration in cell metabolism, activation of mechanisms that protect against oxidative stress, toxic response and finally cell death. Many papers report effects of nano and microparticles on lipid and protein oxidation, changes in cell membrane fluidity, alterations of proteins and of DNA.  

The aim of our work was to study deviations in molecular composition between normal digestive gland tissue and digestive gland tissue of WO_x nanofibers (nano-WO_x) fed animals and reveal mechanism of nano-WO_x effect on digestive gland cells of a model organism in vivo.  

The model organism we used was a terrestrial invertebrate Porcellio scaber (Isopoda, Crustacea). The advantage of using this organism is the possibility of directly correlating the actual exposure dose to nanofibers and observed effects at different levels of biological organization. The feeding parameters are an integrated organism-level response, appropriate evidence of the effects of different chemicals at organism level. Cellular and biochemical analyses indicate cell level evidence after exposure to chemicals or nanoparticles and to a certain degree their mode of action. Digestive gland cells (hepotopancreas) of terrestrial isopods which combine the functions of pancreas and liver in vertebrates are preferred tissue to study effects of substances with unknown and untargeted action in digestive system.
In this study we used FTIR microspectroscopy to study the type and level of digestive gland cell alterations due to ingestion of WO\textsubscript{x} nanofibers. It was expected that comparison of the FTIR spectra of control and nano-WO\textsubscript{x}-fed test animals will indicate the biological consequences of ingestion of nanofibers.

**Experimental**

**Chemicals**

Acridine orange (AO), ethidium bromide (EB), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl\textsubscript{2}), glucose and 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), were purchased from Merck. Cobalt (II) chloride hexahydrate (CoCl\textsubscript{2}.6H\textsubscript{2}O), 99.9% (metal basis) was purchased from Alfa Aesar Johnson Matthey Company. The WO\textsubscript{x} nanofibers were synthesized at Jozef Stefan Institute, Condensed Matter Physics Department. Tungsten powder (99.9%), WO\textsubscript{3} powder (99.9%) and Iodine (99.8%) were purchased from Sigma-Aldrich.

**Model organisms**

Terrestrial isopods (*Porcellio scaber*, Isopoda, Crustacea) were collected during July 2010 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 ± 2 °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 moulting or the presence of marsupia were observed, the animals were not included in the experiment in order to keep the investigated population physiologically as homogenous as possible.
The digestive system of the terrestrial isopod *P. scaber* is composed of a stomach, four blind-ending digestive gland tubes (hepatopancreas) and a gut. 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.

**Synthesis and characterization of WO$_x$ nanofibers**

The WO$_x$ nanofibers were synthesized by a chemical transport reaction$^{14}$ 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 produced nanofibers were studied using electron transmission microscopy 200 keV Jeol 2010F, scanning electron microscopy (FE-SEM, Supra 35 VP, Carl Zeiss). XRD spectra were recorded with an AXS D4 Endeavor diffractometer (Bruker Corporation, Karlsruhe, Germany), with Cu Ka1 radiation and a SOL-X energy dispersive detector with the angular range of 20 from 5° to 75°, a step size of 0.04° and a collection time of 3 to 4 s.

**Food preparation**

In this study the animals consumed particles applied in a suspension to 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 WO$_x$ nanofibers were suspended in distilled water before each experiment to obtain the final concentration of 5000 µg nano-WO$_x$/ml. To diminish agglomeration of nanofibers in distilled H$_2$O the suspension was sonicated in an ultrasonic bath for 1h and mixed using a vortex mixer before brushing it on the leaves. In the control group, the leaves were treated only 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 5000 µg nano-

WO$_x$ per gram (dry wt) of leaf and left until dry.

**Experimental setup**

Each individual animal was placed in a 9 cm Petri dish. A single hazelnut leaf segment treated with distilled water or nano-WO$_x$ suspension (5000 µg/g of WO$_x$) and 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 ($\geq$80%), temperature (21±1°C) and light regime (16:8h light: dark photoperiod). After the exposure, the animals were anaesthetized at low temperature and then decapitated and their digestive glands isolated and subsequently used for different analyses.

**Feeding parameters, weight change and survival**

After 7 days of exposure of the animals to treated leaves, the faecal pellets and leaves were removed from the Petri dishes, dried at room temperature for 24 h and weighed separately. The feeding rate of isopods was calculated as the mass of leaves consumed per animal’s wet weight per day. The food assimilation efficiency was calculated as the difference between the mass of consumed leaves and mass of faecal pellets divided by the mass of consumed leaves. The weight change of an animal was the difference in its mass from the beginning to the end of the experiment.

**Digestive gland cell membrane stability assay**

The cell membrane stability was tested with the modified method previously described by Valant et al.$^{15}$ 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 examined by an Axioimager.Z1 fluorescent microscope (Zeiss) and
photographed 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 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. The cell membrane integrity was assessed by examination of the micrographs. Photographs of intact digestive glands were examined by the same observer twice at intervals of at least 24h. The integrity of cell membrane was assessed visually and classified on the basis of a predefined scale from 0 to 9. From preliminary experiments, it was concluded that the non-treated (control) animals show <5% of nuclei stained by EB, while severely stressed animals have up to 100% of EB-stained nuclei. The <5% of hepatopancreatic tubes stained with EB were classified as 0, and those with the highest proportion (>95%) of EB stained nuclei as 9.15 Our previously published results have demonstrated that in animals in good physiological condition from a stock culture, the digestive gland cell membrane stability value was higher than 2 in only 5% of animals and this was taken as a benchmark.15 The cell membranes are considered to be destabilised when the value is higher than 2.

**FTIR imaging**

For FTIR imaging, digestive glands were shock-frozen in liquid N₂, using tissue-freezing medium (Jung Tissue Freezing Medium, Leica). 15 µm thick samples were sectioned using a Leica CM3050 cryotome with the temperature of the microtome head and chamber maintained between -25 °C and -20 °C. The sections were then placed onto CaF₂ IR transparent windows 2 mm thick.

FTIR measurements were carried out at the infrared beamline SISSI (Synchrotron Infrared Source for Spectroscopy and Imaging) of Elettra Synchrotron laboratory16 using the Vertex 70 interferometer coupled with Hyperion 3000 Vis-IR microscope. Both the interferometer and microscope were purged with N₂. The IR images were acquired in
transmission mode using the bidimensional Focal Plane Array (FPA, 64X64 pixel) detector, averaging 256 scans per spectrum. Each FPA image is composed by 4096 spectra and by using a 15X condenser/objective (NA=0.4), a ~170X170 µm² sample area was imaged achieving a pixel resolution of about 2.6 µm. Each image was pre-processed by running the atmospheric compensation routine of OPUS 6.5 (Bruker Optics GmbH, Ettlingen, Germany) in order to minimize spectral contributions from water vapour and carbon dioxide. The simpler FTIR image is generated by integrating a specific spectral band or a spectral region for each image pixel, following univariate analysis. The integration results are then plotted in 2D using a colour scale and providing information on the distribution of a functional group within the sample, and consequently of the bio-macromolecules that contain it (Figure S1A,B, Supporting Information). Figure S1B (Supporting Information) was obtained following this procedure, integrating the 1720-1480 cm⁻¹ spectral region, which contains the most intense bands of cellular proteins, Amide I and II (Table S1, Supporting Information). The spectral band assignment was done in accordance with published recommendations (listed in Table S1, Supporting Information). The spectral bands relevant for this manuscript and their assignment are reported in Table S1, Supporting Information.

Penetration of the tissue-freezing medium (TFM) into the sample is usually minimal and dependent on the tissue type. Typically, the TFM could be detected as an additional thin layer surrounding the tissue. However, the TFM penetration within the sample was presumed to be different between different specimen regions and among samples, depending also upon dragging effects during cutting. Figure 1 shows the spectra of the tissue freezing medium (black dotted line), of a peripheral and central point of the sample control K2, black and grey continuous lines, respectively. In particular, a strong and sharp tissue freezing medium peak centred at 1116 cm⁻¹ could affect the spectral profile below 1200cm⁻¹. The Jung TFM has vibrational features that overlap with some characteristic spectral bands can impose
some limitation to the diagnostic potentials of the technique: its contribution is clearly visible in the peripheral region spectrum (black continuous line) while it is not detectable in the central part of the same sample. Consequently, a procedure was developed for subtraction of the TFM contribution from the sample vibrational pattern.

We developed a chemometric approach based on the HyperSpecJSS program (http://hyperspec.r-forge.r-project.org). The routine steps are presented schematically in Figure 2 for the control sample K1 (see optical image 2A) and listed as follows: i) Hierarchical Cluster Analysis (HCA), Euclidean distances & Wards’ algorithm on absorbance spectra was carried out in the spectral region 1720-1480 cm\(^{-1}\) (FTIR image of protein distribution in Figure 2B) and the cluster centroid of the freezing medium was identified. It corresponds with the sampled region outside the specimen, black in Figure 2C, ii) The tissue freezing medium distribution was evaluated by integrating the characteristic tissue freezing medium band centred at 1116 cm\(^{-1}\) as height integral (Figure 2D). The integral intensities over the image were normalized to 1 in order to generate the matrix of subtraction coefficients. iii) The TFM spectrum was subtracted pixel by pixel from the sample spectrum by applying the subtraction coefficients obtained in the previous step. The procedure was repeated twice per sample (Figures 2E, F).

Images corrected for the TFM contribution were analysed independently in order to highlight the biochemical diversity between central and peripheral sample regions. The aforementioned regions have been discriminated against by applying HCA in the spectral region 3050-2800 cm\(^{-1}\) on vector normalized absorbance spectra (Euclidean distances & Wards’ algorithm). Comparing optical and FTIR images, it was deduced that apical and basal parts of the epithelium represent the peripheral, while the central part of epithelium, located around nuclei formed the central region (Figure 2I). More than 6000 spectra, divided between
peripheral and central regions, have been selected and further analysed following the
procedure described above.

**Scanning electron microscopy (SEM) and Energy dispersive X-ray analysis (EDX)**

After the feeding experiment, animals were decapitated and the hepatopancreas was
isolated and immediately transferred with tweezers to the fixative containing 2.5%
gluteraldehyde, 0.4% paraformaldehyde and 0.1M sodium phosphate buffer (pH 7.2). After
primary aldehyde fixing, digestive glands were put in 1% osmium tetroxide and stained with
TOTO (thiocarbohydrazide/osmiumtetroxide/thiocarbohydrazide/osmiumtetroxide) conductive, a method previously described by Leser et al.\textsuperscript{21} The fixed hepatopancreas glands were dehydrated in absolute alcohol and dried with hexamethyldisilizane (HMDS). The dry samples were mounted on holders and sputter coated with gold-palladium (Sputter coater SCD 050, BAL-TEC).

Samples were investigated by field emission scanning electron microscopy (SEM; Jeol JSM-6500F, at the Institute of Metals and Technology in Ljubljana). Energy dispersive X-ray analysis (EDX) was used to analyse the chemical composition of selected parts of the epithelial surface (EDX/WDX Oxford Instruments INCA, Jeol JSM-6500F, at the Institute of Metals and Technology).

**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 non-parametric Mann-Whitney U test. All calculations were performed with Statgraphics Plus 4.0.
Results

Characterization of WO\textsubscript{x} nanofibers

The WO\textsubscript{x} fibers were grown as single crystalline fibers with high aspect ratio (Figure 3A). While their diameter typically did not exceed 100 nm, their length was on the millimetre scale. An electron diffraction pattern taken on a single fiber (Figure 3B) corresponds to the W18O49 phase. The [1\bar{2}1] zone axis is shown. X-ray diffraction confirms the monoclinic W18O49 phase (JCPDS-71-2450). Impurities of other WO\textsubscript{x} phases are possible with quantities below the detection limit of 2-3 %.

Feeding parameters, weight change and survival

Weight, survival and feeding parameters were not affected when animals were exposed to leaves dosed with WO\textsubscript{x} nanofiber suspension, providing nominal concentrations of 5000 µg nano-WO\textsubscript{x}/g of leaf.

Digestive gland cell membrane stability assay

In control group, 90% of animals had a cell membrane stability of, nominally 2 (Figure 4). In 10% of exposed animals, it was slightly higher and assigned the value 3. We ascribe this to suboptimal experimental conditions.

In animals fed on food dosed with nano-WO\textsubscript{x} the stability of the digestive gland cell membrane was affected in almost 20% of animals, while 10% of them had severely affected cell membranes (Figure 4).

FTIR imaging

The average spectra of peripheral and interior regions for each sample as obtained from HCA have been compared by verifying their spectral similarities. In particular, we
analysed differences between control and exposed samples in the peripheral region, the apical part of the digestive gland epithelia, and between controls and exposed samples in the central part of the epithelia, which is the region between the apical and the basal layer of the cells. The analysis did not highlight any significant differences between the control and nano-WO$_x$ treated peripheral regions that are also those mostly affected by tissue freezing medium penetration. In contrast, following nanoparticle ingestion noticeable biochemical alterations were found in the internal sectors.

Figures 5A, B show the average spectra of central parts of the WO$_x$ and control samples, as obtained upon standard vector normalization on the 3000-2800 cm$^{-1}$ range. In order to highlight compositional modification affecting the samples following ingestion of WO$_x$ NPs, several spectral regions have been integrated. Since inhomogeneity in the sample thickness, both within and between samples, can affect the integral values, integral ratios have been considered. Second derivatives of average spectra are shown in Figures 5C, D, and have been considered for highlighting structural differences among the samples.

The ratio of area integrals between 1720-1480 cm$^{-1}$ and 3000-2800 cm$^{-1}$ decreased significantly upon ingestion of nanofibers, from 3.536±0.033 to 2.1563±0.221, revealing a remarkable decrease in the protein to lipid content (Table S1, Supporting Information). However, no significant differences in either relative intensity or energy were detected for methyl and methylene stretching bands. Asymmetric and symmetric -CH$_3$ and -CH$_2$ stretching bands were found at 2960±2, 2873±2, 2925±2 and 2852±2 cm$^{-1}$, respectively, in both control and treated samples and their integral height ratio did not change upon treatment. Conversely, an upshift of both vinyl (3007 - 3012 cm$^{-1}$) and methine (2893 - 2896 cm$^{-1}$) moieties was evident upon treatment of nanofibers, followed by the downshift of the carbonyl ester stretching band from 1743 to 1739 cm$^{-1}$. No protein structural variations were detected upon nano-WO$_x$ ingestion. Amide I and Amide II components remained constant and contribution
from α-helix (AmI, 1660 cm$^{-1}$; AmII, 1545 cm$^{-1}$), random coil (AmI, 1640 cm$^{-1}$) and β-sheet (AmI, 1688/1613 cm$^{-1}$) structures were found for both control and nano-WO$_x$ treated samples.

The asymmetric bending mode of methyl groups and the deformation of methylene moieties was centred at 1457 cm$^{-1}$ in control samples, while the methylene ($\nu_{\text{asym}}$CH$_3$, 1464cm$^{-1}$) and methyl ($\nu$ CH$_2$, 1453cm$^{-1}$) contributions could be distinguished in WO$_x$ treated samples. The symmetric bending mode of methyl groups reflected the former trend, splitting from 1394 to 1382 and 1399 cm$^{-1}$ upon ingestion of NPs. The ratio of the bands centred at ~1740 and ~1464 cm$^{-1}$ is much higher for treated samples (0.692±0.105) than for the controls (0.378±0.069). The ratio between ~1464 to ~1400 cm$^{-1}$ changed from 0.600±0.062 to 1.187±0.057. The asymmetric phosphate band had 2 major components, centred at 1217 and 1232 cm$^{-1}$ in both control and treated samples, but the former became much weaker upon treatment.

**Scanning electron microscopy (SEM) and Energy dispersive X-ray analysis (EDX)**

Digestive glands of animals fed with food dosed with 5000 µg nano-WO$_x$/g of food were prepared for scanning electron microscopy. Fiber-like structures were observed on the surface of some cells of digestive glands (Figure 6A). We analysed these structures with EDX and the chemical composition reveals the presence of tungsten (Figure 6B, C).

In 25% of animals these irregularly shaped structures were found to be thrust into the cells. Such structures have never been found in control animals or observed during our previous research of morphological characteristics of digestive gland cells. The EDX analyses indicate elevated amount of tungsten approximately 2.4 wt% in these structures as shown in Table 1.
Discussion

The application of synchrotron based FTIR microspectroscopy to studies of molecular alterations in the digestive gland cells due to ingestion of WO$_x$ nanofibers by model invertebrate organism *P.scaber* have provided new insight into cellular response to nanofibers *in vivo* that can be complemented with the toxicological and cytological results and the inspected surface status of digestive gland epithelium by scanning electron microscopy and energy dispersive X-ray spectrometry.

In presented study classical toxicological parameters (weight change, survival and feeding rate) were not affected when animals were exposed to leaves dosed with WO$_x$ nanofiber suspension, providing nominal concentrations of 5000 µg nano-WO$_x$/g of leaf. After seven days of feeding with nano-WO$_x$ dosed food the stability of the digestive gland cell membrane was affected in almost 20% of animals.

The FTIR spectral imaging revealed several significant differences in molecular composition of digestive gland epithelium between control animals and animals exposed to nano-WO$_x$. Hierarchical cluster analysis of all analysed sections of gland epithelium indicated that the epithelium could be divided into two distinct regions: the central one around the nuclei, and the remainder, which is termed a peripheral region. Since distinct differences between the control and nano-WO$_x$ treated digestive gland epithelium were observed only in the central region, only the central regions will be considered here.

The most evident compositional difference between control and WO$_x$ treated samples was found in the lipid to protein ratio. Compared to control cells, the central region of treated cells is enriched in proteins with respect to lipids. This could be interpreted either as increased lipid metabolism due to nano-WO$_x$ ingestion or increased protein synthesis. Since there are no differences in the Amide I and Amide II regions indicating proteins, we assume that the
difference in the protein/lipid ratio may be attributed to altered lipid metabolism. Similar modifications of lipid/protein ratio have been reported in Gaigneaux et al., where the authors suggest that an increase of the ratio occurs in multiresistant cells.

The methyl to methylene ratio is comparable within the standard deviation of measurements between control and nano-WO$_x$ treated samples, and reveals that the average length and ramification of acyl chains of lipids, and phospholipids in particular, was unaffected by the ingestion of nanofibers. The energies of the methyl and methylene stretching bands are also unaffected. The positions of the signals corresponding to the –CH$_2$ and –CH$_3$ moieties provide information regarding the packing characteristics of the acyl chains, which in turn may be related to the fluidity of membrane. Our spectroscopic evidence supports the hypothesis that neither the membrane composition nor structure change as a consequence after nano-WO$_x$ consumption affected membrane fluidity-rigidity. We have not observed any variations in saturation level of lipids but the observed upshift on both vinyl and methine stretching indicates that the unsaturated acyl chains are to a certain extent affected by the ingestion of nanofibers.

The FTIR data also show that the carbonyl-to-methyl/methylene ratio (1740/1464 cm$^{-1}$) increased upon nanofibers ingestion. Levine et al. and Palaniappan and Pramod interpreted a similar observation as a sign of lipid peroxidation. In our study the downshift and relative increase of the carbonyl band at ~1740 cm$^{-1}$ and the decrease of the methyl/methylene to carbonyl ratio (1464/1400 cm$^{-1}$) indicates that oxidative processes are taking place. However, we have no indication for changes in the secondary structure of the proteins, which excludes occurrence of protein oxidation.

Comparing the results for control and nano-WO$_x$–treated samples we also found some alterations in the spectral range between 1300 and 1000 cm$^{-1}$, characteristic for nucleic acids.
The asymmetric stretching band of the $\text{PO}_2^-$ in the backbone of nucleic acids shifts to higher wave numbers upon nano-WO$_x$ ingestion (from 1226 to 1232 cm$^{-1}$). This trend can be a consequence of a partial reorganization of the nucleic acid structure. Similar FTIR results, shifts from 1225 to 1238 cm$^{-1}$, were also by Whelan et al.$^{25}$ who explain it as an indication of transitions from the B form to the A form of DNA. These authors interpreted this DNA change as a response of different cell types to dehydration. In addition, there are also literature reports suggesting that the B $\rightarrow$ A transition of DNA might play role in the resistance of DNA to potential damage caused by heat, desiccation and toxic damage.$^{26}$

Same as in our study Dovbeshko et al.$^{27}$ have also observed changes in spectral range between 1300 and 1000 cm$^{-1}$. They interpreted asymmetrical stretching vibration (1225 cm$^{-1}$) together with symmetrical stretching vibration (1088 cm$^{-1}$) in phosphate groups to be a consequence of damage caused to cells by irradiation. That may be related to spatial changes in the positions of the phosphate groups in the RNA helix.

In parallel to FTIR and classical toxicity analyses, additional information was provided by SEM inspection of the surface of digestive gland epithelium. In 2 of 8 investigated animals, rod like structures containing tungsten have been found, thrust into the apical part of a few epithelial cells. This indicates that the ingested WO$_x$ nanofibers have the potential to interact with individual cells and may result in time in a deleterious effect. This phenomenon has been well studied in the case of asbestos. Wang et al.$^{28}$ report asbestos fibres insert into pleural mesothelia cells, inducing chromosomal changes by direct biological or mechanical damage. Similarly, some well knew toxic effect of asbestos nanofibers have also been found in carbon based nanotubes.$^2$

The FTIR results obtained in our study are in agreement with toxicological and cytological measurements, which indicate that ingestion of nano-WO$_x$ does not affect severely
the cell membrane stability and feeding behaviour. However, FTIR analyses clearly reveal that the cells are disturbed and respond to the presence of nano-WO$_x$ in the digestive juices.

We succeeded to show that ingested WO$_x$ nanofibers activate some cellular mechanisms that may act as a protection against unfavourable conditions. Changed protein to lipid ratio, lipid peroxidation and structural changes of nucleic acids we interpret as responses indicative of non-homeostatic state before oxidative stress and toxic responses are evidenced. To what extend and if at all they are nanoparticle or nanofibres specific is a matter of further research.

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Notes

The authors declare no competing financial interest
Tables

Table 1: EDX elemental composition

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Table 2: EDX elemental composition of spectrum 1 observed in area showed in Figure 4B., results in wt.% with some other elements present in cell.

Figures
Figure 1. Spectra of tissue freezing medium used in samples for FTIR analyses. FTIR spectra of pure tissue freezing medium (dotted spectrum), of a sample point containing a negligible amount of tissue freezing medium (gray spectrum) and a sample point where the tissue freezing medium contribution is clearly visible from the sharp band centred at 1116 cm$^{-1}$ (black spectrum).

Figure 2. The routine steps of chemometric approach in control sample after FTIR analyses. A) Optical image of the control sample K1. B) Chemical FTIR image of the sample obtained integrating the protein spectral region 1720-1480 cm$^{-1}$ (peak area). C) HCA results that show that the tissue freezing medium cluster centroid is the number 1, - the black region outside the sample. D) TFM distribution obtained by integrating the sharp characteristic TFM band centred at 1116 cm$^{-1}$ (peak massif). Clearly, the TFM penetrates the sample. E,F) TFM distribution after the first and second subtraction. G,H) Chemical FTIR image of the sample
obtained integrating the spectral region of nucleic acids and sugars 1300-900 cm\(^{-1}\), before and after subtraction. The effects of subtraction are particularly clear in the external and peripheral sample regions. The subtraction procedure does not affect the protein or the lipid distribution obtained by integrating the spectral region 3000-2800 cm\(^{-1}\) (data not shown). I) Hyperspectral image of the control sample K1 obtained by HCA on vector normalized spectra after TFM subtraction in the spectral region 3000-2800 cm\(^{-1}\).

**Figure 3.** Transmission electron microscopy (TEM) of WO\(_x\) nanofibers. A) TEM micrograph of W18O40 nanofibers; B) A diffraction pattern taken on a single fiber showing [1\(\bar{2}1\)] zone axis.
Figure 4. Digestive gland cell membrane stability of control and nano-WO₃ exposed animals. Percentage of animals in each exposed group, with different degrees of destabilised cell membrane, assessed visually and classified from 0 to 9 according to the predefined scale as described in Materials and Methods. A digestive gland cell membrane stability value of 2 or less denotes animals which 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.
Figure 5. Average spectra of the central regions of digestive gland tissue. A,B Average spectra of the central regions of WOx treated (grey) and control (black) samples. C,D Second derivative of the average spectra of treated (grey) and control (black) samples, respectively; Savitzky-Golay algorithm, 17 smoothing points.)
Figure 6. Scanning electron microscopy (SEM) and Energy dispersive x-Ray (EDX) composition of fiber-like structures in digestive gland. Surface of digestive gland epithelium of animal fed with tungsten nanofibers for 7 days with fiber like structures found in one of the cells (A). Digestive gland epithelium cell with thrusted fiber like structures where EDX spectrum was taken (B). EDX spectra of observed area (pointed in figure B) (C).

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Supporting Informations

Spectral band assignment where the relevant positions and assignments of the spectral bands are reported and listed in Table S1. In Figure S1 is optical and Fourier transform infrared (FTIR) image of control sample.

References


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