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Enabling Science through European Electron Microscopy

Materials for Health

Deliverable D9.1 – version 1.2

Estimated delivery date:30.04.2020

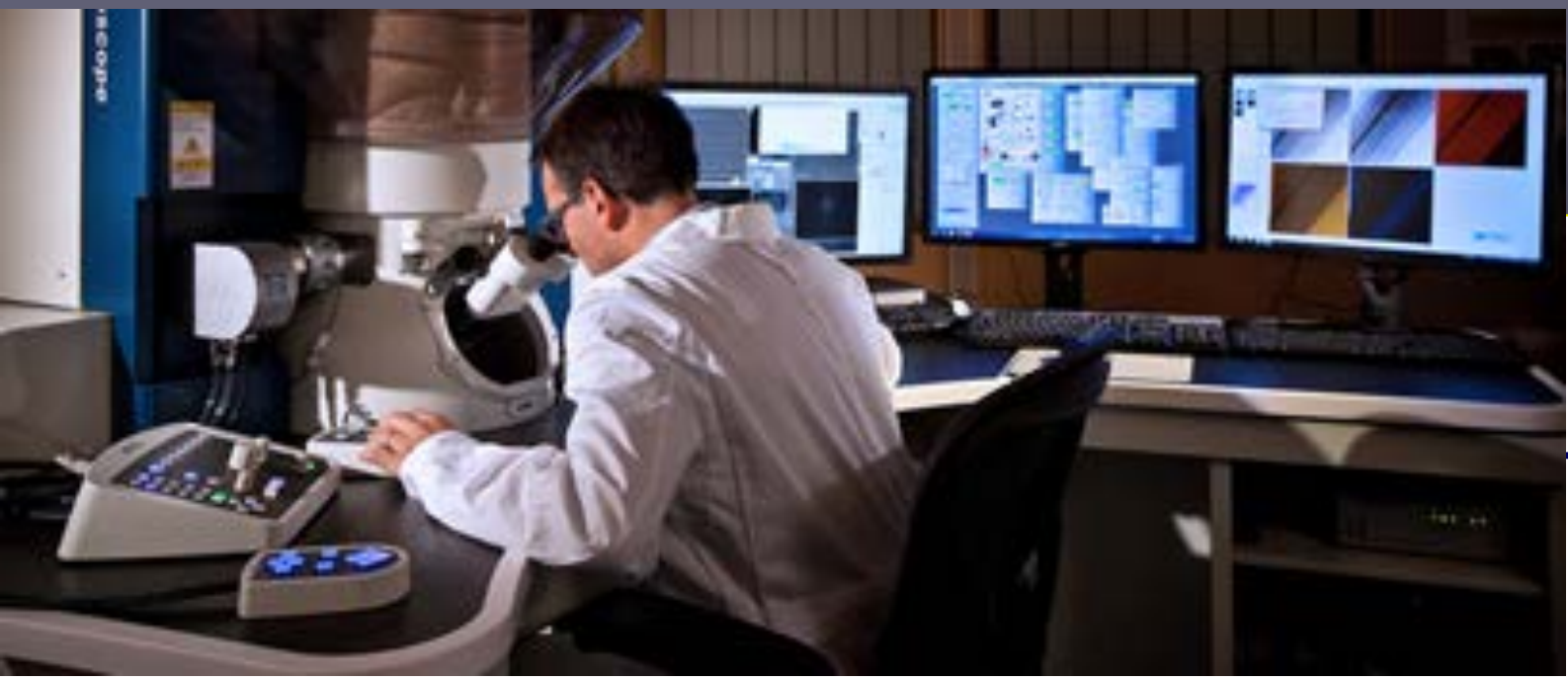
Actual delivery date: 30.05.2020

Lead beneficiary: UOX

Person responsible: Angus Kirkland

Deliverable type: ☒ R ☐ DEM ☐ DEC ☐ OTHER ☐ ETHICS ☐ ORDP

Dissemination level: ☒ PU ☐ CO ☐ EU-RES ☐ EU-CON ☐ EU-SEC



THIS PROJECT HAS RECEIVED FUNDING FROM THE EUROPEAN UNION'S HORIZON 2020 RESEARCH AND INNOVATION PROGRAMME UNDER GRANT AGREEMENT NO **823717**

Grant Agreement No:	823717
Funding Instrument:	Research and Innovation Actions (RIA)
Funded under:	H2020-INFRAIA-2018-1: Integrating Activities for Advanced Communities
Starting date:	01.01.2019
Duration:	48 months

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Revision history log

Version number	Date of release	Author	Summary of changes
V0.1	25.05.2020	AIK	First draft
V0.2	27.05.2020	AIK	Minor amendments
V1.1	28.05.2020	Lucie Guilloteau	General review
V1.2	29.05.2029	Peter A van Aken	Final check and approval

Executive Summary

This report summarises three projects carried out within the Materials for Health workpackage and forms the first of a series of such reports.

Investigations of Rodent Teeth (STU)

Mammalian teeth are a composite product of biomineralization, where simple inorganic and organic components are arranged in optimally designed nanoarchitectures. Such materials show outstanding performance and fulfill different important functions in animal bodies. The constantly growing incisors of rodents are a perfect example of such natural complex organic-inorganic material. The bulk of the tooth, dentine, consists of elongated hydroxyapatite platelets that are closely linked to organic material, and is intersected by numerous dentinal tubules.

We have been investigating the microstructure and chemical composition of constantly growing incisors of different selected rodent species with the aim to identify the mechanism responsible for the teeth growth. Preliminary studies show the presence of unprecedentedly high amounts of Mg in only the continuously growing incisors but not in molars of chosen rodent species, coypu (*Myocastor coypus*).

TEM sample preparation of inorganic/organic composite materials, such as dentine, is a very delicate and demanding task, because of the co-existence of materials with inorganic and organic components that possess dissimilar mechanical, physical, and chemical properties. It is well known to be the most limiting factor for successful investigations in electron microscopy, especially when materials with different properties and composition co-exist (e.g. organic-inorganic composite materials). Our previous studies have shown that for the preparation of dentine, especially when amount of DT is relatively high, cutting thin lamellae via ultramicrotomy gives the best results. We are constantly working on the improvement of sample preparation of such materials.

Detection of Pt in Cells (UOX)

Work performed by Alex Sheader and Pete Nellist (OXF) in collaboration with Roland Fleck and Sarah Flatters (Kings College London).

Oxaliplatin is a first-line chemotherapeutic used to treat a number of cancers. Its structure contains a single atom of platinum, which preferentially binds to DNA and causes cell death. However, there are numerous side effects associated with oxaliplatin treatment. One of the most clinically challenging of these is oxaliplatin induced peripheral neuropathy (OIPN). This causes pain and numbness in the extremities, which at present cannot be alleviated or prevented. OIPN is a primary reason for discontinuation of an otherwise effective chemotherapeutic regimen, which can impact on patient quality of life and survival.

The scanning transmission electron microscope has been used successfully to image oxaliplatin for clinical formulation in Sheader et al (2018). This work found that the oxaliplatin drug molecules form small clusters, in contrast to another related Pt-based chemotherapeutic cisplatin (see Figure 1).
As part of an effort to understand how peripheral neuropathy arises during oxaliplatin treatment, it is necessary to be able to locate platinum at the sub-cellular level while maintaining ultrastructural context. Existing techniques such as mass spectrometry and nano-secondary ion mass spectrometry lack either the spatial resolution required, or are prohibitively slow (thus limiting the number of cells

and samples which can be examined.) In contrast, scanning transmission electron microscopy (STEM) is well-suited to detecting heavy elements within a low atomic number matrix.

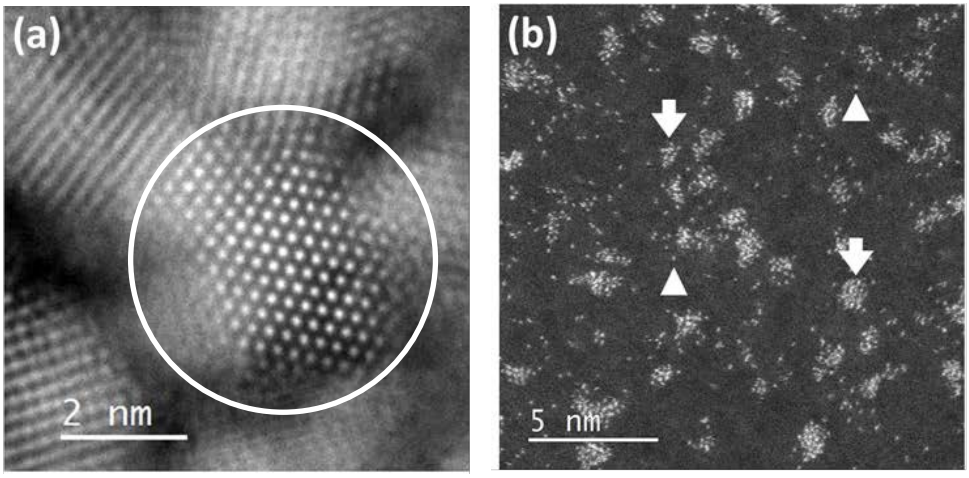

Figure 1: (a) a platinum nanoparticle contained in the Pt-based chemotherapeutic cisplatin. (b) non-metallic Pt nano clusters in the sibling drug oxaliplatin do not form nanoparticles, despite the similarities in drug structure.

Following biopsy of the dorsal root ganglia from oxaliplatin-treated rats, cryosectioning and subsequent thawing provided sufficiently thin electron-transparent samples for use in the STEM. Low magnification survey images (see Figure 2) allowed confirmation both that the section contained the correct cell type, and that the ultrastructure was well preserved despite the use of only minimal fixation.

Images of this nature allowed individual cells to be searched for regions with enhanced image contrast. As all were high-angle annular dark field images, any change in contrast was directly attributable to higher average atomic number in that region. The nature of this contrast means that a single atom of platinum, with an atomic number of 79, will scatter more than 80 times as strongly as a single carbon atom (Z=6).

Bright features observed in the DRG neuronal cell body cytosol were found to be aggregations of platinum, occurring due to treatment of the rats with oxaliplatin; see Figure

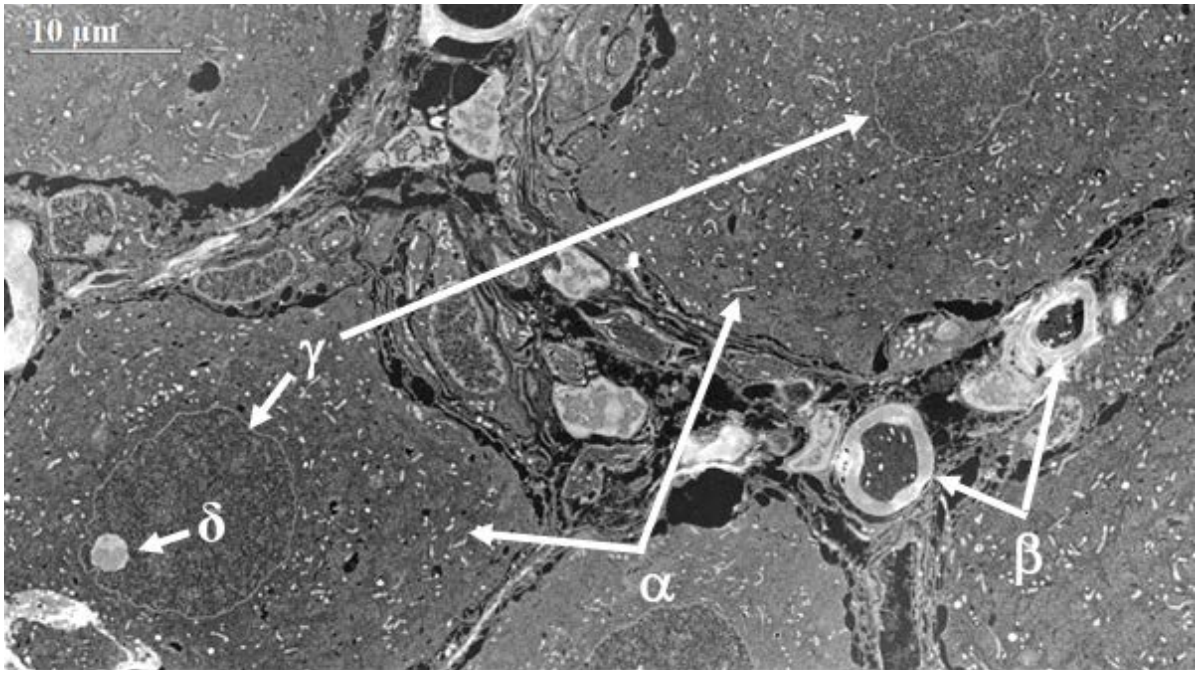

Figure 2: Low-magnification screening images confirm that sections have been cut from the correct region of the DRG; alpha and beta are neuronal cell bodies and myelinated axons respectively. These images also confirm that cell ultrastructure has been preserved; gamma shows preservation of the nuclear membrane and nucleus, and delta shows the cell nucleolus.

3. Further increasing the magnification allowed the visualisation of single atoms. By combining knowledge about the detector geometry and sensitivity, it is possible to represent this image as a fraction of the incident electron beam rather than as arbitrary detector counts. The amount of scattering from a single atom can then be directly compared with simulation, establishing the single atoms we observed were indeed platinum.

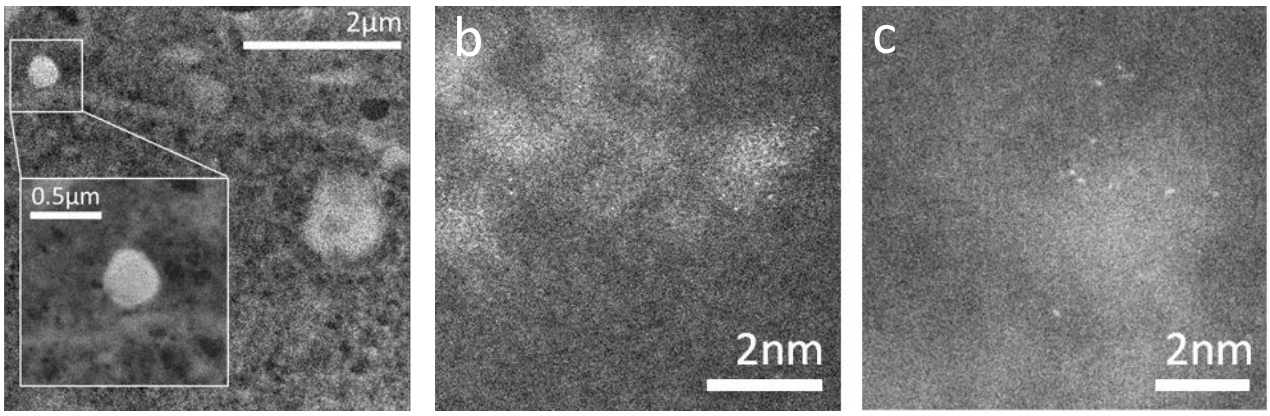

Figure 3: (a) features observed on the exterior of the nuclear membrane (inset) are found to contain many clusters of single atoms (b, c). Comparison with simulation confirms that these are platinum atoms, introduced to the animal by treatment with oxaliplatin.

The amount of platinum in an aggregation cluster was then measured. As HAADF STEM is an incoherent imaging technique, the scattering induced by the platinum aggregation may be treated as a linear sum of the scattering from individual Pt atoms. We calculated an average cluster was 3.5nm in size, and contained approximately 1000 atoms. This is equivalent to 10^{-21} mol, and well below the detection sensitivity of conventional mass spectrometry.

Part 2: Quantification of EDX in biology

Energy dispersive x-ray spectroscopy (EDX) makes use of the x-rays produced by an inelastic electron-sample event. These x-rays have characteristic energies which are associated with particular elements. In a biological specimen, which is composed of many elements distributed non-uniformly across different cell types, EDX can be used to map cell composition.

However, quantifying EDX in such samples is a multi-faceted challenge. As many elements are present within a single region, EDX peak overlaps are common. For example, the calcium Kα peak is often convolved with the potassium Kβ peak. Copper (a typical EM grid material) obscures detection of sodium and therefore should be avoided in favour of gold grids, but this introduces additional challenges for sample preparation. In addition, low EDX partial cross-sections mean that trace elements are hard to detect and accurately measure.

Nevertheless, EDX is a valuable tool for examining sub-cellular elemental distributions. The ability to capture information about the whole population of elements within a cell relatively rapidly with high resolution is particularly valuable when it can be combined with the ultrastructural information offered by routine ADF imaging.

Samples of peripheral nerve were prepared by high-pressure freezing, cryoultramicrotomy, and freeze-drying. An example of a peripheral nerve section is shown below in Figure 4a. EDX mapping was performed on myelinated and unmyelinated axons in samples harvested from chemotherapy- and vehicle-treated rats. A typical EDX spectrum can be seen in Figure 4b.

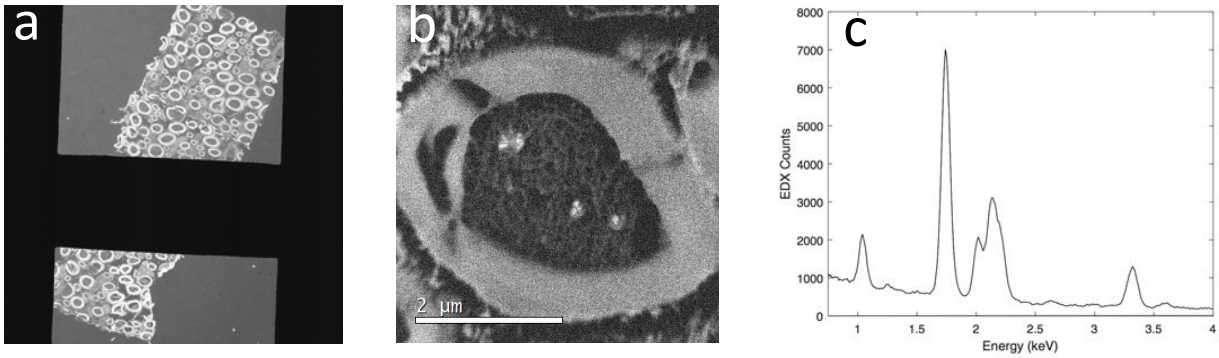

Figure 4: (a) Low magnification of freeze-dried section containing many peripheral nerves. (b) A single myelinated axon (c) EDX recorded from the axon cytosol shows it contains many different elements.

The high resolution of the STEM enables spectral mapping at high resolution. For example, we have shown it is possible to map calcium within a mitochondrial calcium phosphate store (see Figure 5).

Typically in commercial software, EDX spectra are quantified using either k-factors (Cliff & Lorimer) or Zeta-fator (Watanbe) methods. However, both approaches are known to be accurate to no more than 5%, which potentially lacks the ability to detect low concentrations in a multi-element sample.

We are developing an approach based on inelastic cross-sections, which can be easily measured from known standard samples.

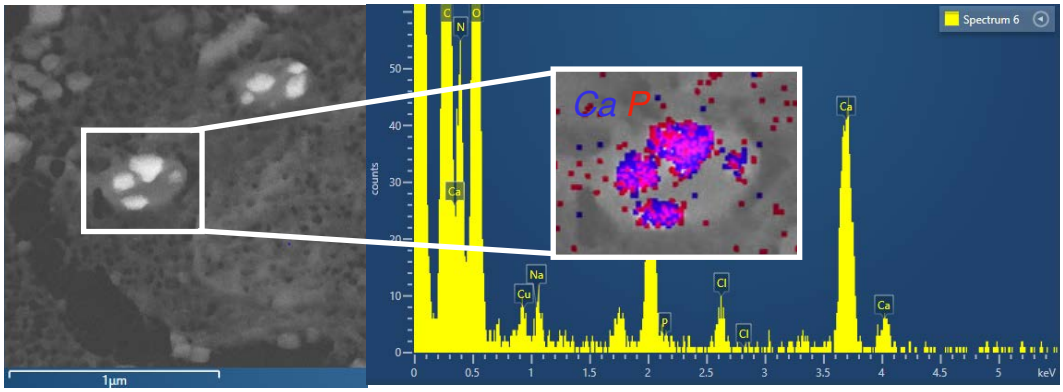

Figure 5: (a) Mitochondria within an axon of rat peripheral nerve. (b) EDX spectral map of mitochondrial CaPO storage granule. (c) EDX spectrum integrated over (b) shows clear Ca peaks.

References

A.A. SHEADER (2018), A.M. VARAMBHIA, R.A. FLECK, S.J.L. FLATTERS, P.D. NELLIST, Observation of metal nanoparticles at atomic resolution in Pt-based cancer chemotherapeutics, Journal of Microscopy, **270**, 92-97.