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Structure and composition of myelinated axons: a multimodal synchrotron spectro-microscopy study

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Abstract

We report elemental mappings on the sub-cellular level of myelinated sciatic neurons isolated from wild type mice, with high spatial resolution. The distribution of P, S, Cl, Na, K, Fe, Mn, Cu was imaged in freeze-dried as well as cryo-preserved specimen, using the recently developed cryogenic sample environment at beamline ID21 at the European Synchrotron Radiation Facility (ESRF). In addition, synchrotron radiation based Fourier transform infrared (FTIR) spectromicroscopy was used as a chemically sensitive imaging method. Finally single fiber diffraction in highly focused hard x-ray beams, and soft x-ray microscopy and tomography in absorption contrast are demonstrated as novel techniques for the study of single nerve fibers.

Key words: myelin, x-ray microscopy, x-ray fluorescence, spectromicroscopy, x-ray tomography

1. Introduction

Myelin is a spiral structure formed by extensions of the plasma membrane of the myelinating glial cells, the oligodendrocytes in the central nerve system (CNS) and the Schwann cells in the peripheral nervous system (PNS) (Quarles et al., 2006). During the myelination process, these cells extend their plasma membrane to form a compacted stack of concentric wraps, the myelin sheath (Lazzarini et al., 2004). While oligodendrocytes are able to myelinate numerous axons, Schwann cells tightly associate with one single fibre and only myelinate one internode (Siegel, 2006). Several structural features characterize myelin (Siegel, 2006). Its periodic structure, with alternating concentric electron-dense and light layers is easily detected by electron microscopy (Kirschner and Hollingshead, 1980). The major dense line (appearing dark in the electron microscope) is formed by the close apposition of the cytoplasmic surfaces of the glial plasma membranes. The intraperiod line (appearing light in the electron microscope) results from the closely apposed outer leaflets of glial membranes. The periodicity of the lamellae depends on the species and neuron types, and it is around 16-18.5 nm in PNS and 15-17 nm in CNS. The typical length of each myelin sheath segment or internode is in the range of 150-200 μm in the CNS and up to 1 mm in the PNS (Kirschner and Blaurock, 1992; Siegel, 2006). Internodes are separated by spaces where myelin is lacking, the nodes of Ranvier. The nodes of Ranvier play a major role in nerve impulse conduction. The myelin sheath around most axons constitutes the most abundant membrane structure in the vertebrate nervous system. Its composition, the richness in lipids and low water content ensures the electrical insulation of axons, and its unique segmental structure enables the saltatory conduction of nerve impulses necessary for fast nerve conduction in the thin fibers (Baumann and Pham-Dinh, 2001), in contrast to the slow propagation of nerve signals along the axon in unmyelinated or demyelinated fibers (Baumann and Pham-Dinh, 2001). Myelin of every mammalian species contains cholesterol, phospholipids, and glycolipids in molar ratios ranging from 4:3:2 to 4:4:2 (Quarles et al., 2006). The importance of myelin for signal conduction is highlighted by its role in a number of different neurological diseases such as leukodystrophies, multiple sclerosis (MS) in the CNS and peripheral neuropathies in the PNS or hypermyelination (tomaculous neuropathy- HNPP) (Suter and Nave, 1999; Chance, 2001).

Important questions related to myelin assembly and its spatial organization, such as the exact three-dimensional sequence of the topological fold

of this organelle, as well as to the chemical composition and distribution of metabolites during biogenesis in the process of myelination are still unknown. To address these questions, a combination of imaging and spectroscopic techniques is needed. More generally, the chemical composition and the distribution in particular of trace elements in neural cells and organelles such as the myelin sheath is of significant interest, e.g. since trace metals are suspected to be involved in several neurodegenerative diseases (Crichton and Ward, 2005). Some of these elements, like Fe, Cu and Mn, induce significant cellular disturbances, e.g. involving oxido- redox reactions, when present in excess concentration, but they are physiologically necessary in trace amounts. On the other hand, macro-elements like Na, P, S, Cl or Ca directly reflect the homeostasis of the cell. These elements are necessary for many functions and are essential for normal biochemical processes in the cell. Only a few techniques allow element imaging on the cellular level (Fahrni, 2007). Since recently, x-rays are used increasingly for trace element mapping of different cells (Bohic et al., 2001), (Bacquart et al., 2007). In view of structure preserving sample preparation as well as of radiation damage, rapid cryogenic cooling and subsequent measurement under cryo-condition is in the moment the best option for x-ray spectro-microscopy (Bacquart et al., 2007).

In this work, we use synchrotron x-ray fluorescence imaging to image the elemental distribution of P, S, Cl, Na, K, Fe, Mn, Cu in freeze-dried as well as cryo-preserved specimen, using the recently developed cryogenic sample environment at beamline ID21 at the European Synchrotron Radiation Facility (ESRF). We present chemical elemental maps at the sub-cellular level of myelinated sciatic axons isolated from sciatic nerves of 20 day old wildtype mice, with high spatial resolution, in view of characteristic differences in the spatial distribution at internodes and node of Ranvier (RN).

In addition, three more imaging modalities using synchrotron radiation are included in this paper to explore to what extent these can be used for studies of single nerve fibers: (i) synchrotron radiation based Fourier transform infrared (FTIR) spectromicroscopy, (ii) single nerve diffraction using hard x-ray beams with a cross section of about 150nm (so-called hard x-ray nanoprobe), and finally (iii) soft x-ray microscopy based on absorption contrast. As shown before (Choo et al., 1996; Szczerbowska-Boruchowska et al., 2007), synchrotron-based FTIR micro-spectroscopy is a useful tool for imaging the organic compounds in neural cells, without any staining/labeling or cutting artifacts. The contrast of this imaging technique is based on the local density of vibrational states characteristic for functional molecular groups

(Stuart, 1996). Synchrotron radiation is indeed the most useful source for IR, as it is a "white" source with a flat broadband distribution over the entire infrared spectral range (from far-IR to near-IR). The high brightness compared to conventional laboratory sources (Duncan and Williams, 1983; Dumas and Tobin, 2003) opens up the opportunity to combine the highest spectral resolution with a spatial resolution at the diffraction limit (usually half the wavelength of the vibrational frequency) (Dumas and Tobin, 2003; Diem et al., 2004). It has been shown that IR and x-ray images can be correlated precisely, with a spatial resolution of about 2-3 microns (Miller et al., 2007). The novel opportunities of highly focused synchrotron x-ray radiation can be used to study the local multilamellar structure in a single nerve fiber in diffraction, translating the classical work of myelin diffraction (Kirschner and Hollingshead, 1980; Kirschner and Blaurock, 1992) on the level of an entire (averaged) nerve to an individual fiber, with the possibility to distinguish characteristic regions like the node of Ranvier from the internode.

While we explore several different contrast modes and imaging modalities for single fiber microscopy, we focus on x-ray fluorescence (XRF) in this paper.

2. Methods

Sample preparation: Single myelinated fibers were isolated from sciatic nerves of 20 days old wild type mice. Immediately after isolation, the tissue was stored in pH 7.4 Na-phosphate buffer (100 mM). "Teased fibers" were prepared on glass slides in Na-phosphate buffer by using two thin needles, and afterwards shortly washed in ammonium acetate buffer pH 7.4, and then transferred to 0.4 μm thick polypropylene film (Novolen 1320, BASF, Mannheim, Germany), attached to the 3 mm Ni-slot grid holder. After transferring to the polypropylene holder, the sample was left for a few seconds to attach to the foil and afterwards quickly blotted with fine filter paper and immediately rapidly frozen in liquid ethane cooled with liquid nitrogen up to -180°C and afterwards stored under liquid nitrogen. Subsequently, two types of preparation schemes were used, one for cryogenic (frozen hydrated) conditions, and one for freeze dried samples. For the first type, samples were stored in liquid nitrogen until starting the experiment with the cryo-setup. In the second case, sample were freeze dried at maximum -100°C for two days (home built apparatus) and over the next two days the temperature was increased slowly to 20°C , and stored in a desiccator over silica gel at

room temperature. Freeze dried samples were used for light microscopy, FTIR microscopy and atomic force microscopy (AFM) at room temperature. For the first XRF experiment performed at 2.9 keV samples were stained for 30 min with 2% OsO₄ before plunge freezing and freeze drying. Most experiments were performed in the vicinity of Ranvier's nodes, after optical in-situ and ex-situ inspection.

X-ray fluorescence microscopy: The spectromicroscopy experiments were carried out at the undulator beamline ID21 of the European Synchrotron Radiation Facility (ESRF) in Grenoble. ID21 is a windowless UHV beamline - continuous with the storage ring vacuum, dedicated for x-ray microscopy and spectromicroscopy (Solé et al., 2007). The beam is monochromatized by a fixed exit double crystal monochromator (Kohzu). The experiment was carried out, at photon energies, $E=2.9$ and 7.2 keV, respectively. Typically, the spot size varies between 0.3 to 1 micron depending on the energy, with a beam flux of the order of $10^8 - 10^9$ photons/sec/Si(111) bandwidth. The rejection of unwanted focusing orders of the zone plate is ensured by the combined use of a central stop and an Order Selecting Aperture (OSA). The sample is aligned in the focal plane of the zone plate and raster scanned in the micro-beam to collect 2D images. Silicon photodiodes are used for transmission measurements. Fluorescence is collected by a high purity germanium detector (HpGe) from Princeton Gamma-Tech and/or a Silicon Drift Diode (SDD) (XFLASH 2001, Röntec). Transmission signal as well as a full fluorescence spectra can be recorded for each pixel of the map. For a second beamtime, the Fresnel zone plate (FZP) was replaced by a Kirkpatrick-Baez (KB) mirror system. In summary, we present results obtained in either of the two following settings: FZP focusing of a 2.9keV beam to a beam size of about $0.3(v) \times 0.5(h)\mu\text{m}$ (setting denoted as FZP- 2.9keV in the following), or KB focusing of a 7.2keV beam to a beam size of about $0.3(v) \times 0.8(h)\mu\text{m}$ (setting denoted as KB- 7.2keV in the following). Two-dimensional analysis of elemental distribution in samples of neural cells and tissues were performed, with typical areas of $50 \times 30 \mu\text{m}^2$ selected for scanning, and typically mapped by steps of $0.2 \mu\text{m}$ (V). For the images shown below, pixel size and scan area are indicated separately in the caption. The areas of interest were selected by means of an on-line optical microscope, normal to the sample face, see Fig.1. Before x-ray measurements, the nerve fibers were always imaged with the in-situ video microscope. The beam flux on the sample was about 3.5×10^8 photons/s for FZP- 2.9keV , and 3×10^{10} photons/s for KB- 7.2keV , respectively, depending on the exact ring cur-

rent. The measurement time (dwell time per pixel) was below or equal to 200 ms per pixel. For the calibration of the fluorescence lines, single and double elemental SXRF thin film calibration standards and NIST standard reference materials (1577b) were used.

Hard x-ray nanoprobe and diffraction: The ID22NI experiment was carried out using the intrinsic monochromaticity of the undulators of about $\Delta\lambda/\lambda \simeq 0.01$. The photon energy was set to $E=17.5$ keV. The beam was focused by an adaptive Kirkpatrick-Baez (KB) mirror system (Hignette et al., 2005). The focus of the KB-mirror system was characterized by translation of an Au stripe of a nano-fabricated test pattern (Xradia, USA) recording both the transmitted intensity by a diode and the Au- L_α fluorescence by a silicon drift detector (Vortex-EX, SII NanoTechnology Inc., USA). The measured focal spot size (FWHM) was 160 nm in the vertical and 146 nm in the horizontal direction, respectively. The total intensity in the focal spot was on the order of 10^{11} photons per second. The sample was positioned in the focal plane of the KB system and translated by piezo stages in the directions perpendicular and parallel to the beam. During the scanning process the fluorescence signal was detected with the silicon drift detector (Somogyi et al., 2005; Tucoulou et al., 2008), parallel to the recorded diffraction or holographic in-line signal using the fast acquisition CCD camera developed at ESRF (FreLON) (Bravin et al., 2003).

Soft x-ray tomography: The images for X-ray tomography were collected at the new BESSY full-field transmission X-ray microscope at the undulator U41 (Guttmann et al., 2009; Heim et al., 2009), using photon energies just below the oxygen edge, i.e., 510 eV. The new optical setup enables high spatial and spectral resolution $\lambda/\Delta\lambda \simeq 9000$ and tomography of cryogenic samples on flat sample Cu-grids using a Gatan model 630 high tilt holder. A single bounce ellipsoidal capillary condenser is used for hollow cone illumination of a $20\mu\text{m} \times 20\mu\text{m}$ field of view (FOV) on the specimen. A high resolution micro zone plate with an outermost zone width of 40 nm was used to magnify the sample onto the cooled back-illuminated soft x-ray CCD camera (Roper Scientific, PI SX1300). The samples were prepared by teased fiber procedure as described above. They were kept in PBS buffer at pH 7.8 and for a few second washed in pH 7.8, 50 mM ammonium acetate buffer to remove all inorganic ions before transferring them to the Cu grids (Gatan holder model 630), coated with a polyvinyl formal (formvar, SPI Inc.) foil. The foils were prepared by placing the grids onto a thin film (thickness in the range of 10 to 30 nm) of formvar spread out on a wa-

ter surface. Parafilm foils were then used to pick up and turn the formvar covered grid, before drying and cutting. The samples were blotted using filter paper sticks before the cryo plunging procedure described above. For tomography, 108 images were collected at 0.5° intervals through 54° of rotation. The projection series was then aligned to a common axis of rotation and a 3-D volume reconstruction was performed using simultaneous iterative reconstruction technique (SIRT) (Messaoudi et al., 2007) by using Image J software (<http://rsbweb.nih.gov/ij/>). Surface reconstruction and volume segmentation were not performed due to lack of axial resolution by the missing wedge.

FTIR micro-spectroscopy: The FTIR measurements were carried out at the FTIR specro-microscopy station of ID21. The IR microspectroscopic maps were collected in reflection mode using an Infrared microscope (Thermo Nicolet Nexus) coupled to a FTIR spectrometer (Thermo Nicolet Continuum). The IR microscope is equipped with a motorized sample stage and a liquid nitrogen cooled mercury cadmium telluride (MCD) detector. Most of the analysis and maps were achieved with a project area on the sample, of $3 \times 3 \mu\text{m}^2$ and step size of $2 \mu\text{m}$, and each spectrum was acquired after 128 accumulations at 4 cm^{-1} spectral resolution.

Atomic force microscopy (AFM): For the AFM experiments, identical freeze dried samples and/or sample preparation were used. AFM images were recorded in tapping mode using a BioScope instrument with NanoScope IIIa controller (Digital Instruments, Santa Barbara, CA, US). Oxide-sharpened silicon nitride AFM probes (Digital Instruments) with normal spring constants 0.06 and 0.12 N/m were used. The force was kept minimal during scanning by routinely decreasing it until the tap left the surface and subsequently increasing it slightly to just regain contact. The scan rate was 0.5 to 2 lines per second for the tapping mode of operation. All images with 512×512 pixels were analyzed with non-commercial software WSxM (Horcas et al., 2007).

Scanning electron microscopy (SEM): Uncoated samples, prepared as described above with cryo-plunging, but without freeze drying were used for the investigation by a field emission SEM using a LEO 1530 Gemini instrument at 1 kV under cryo-conditions. In order to avoid dehydration artifacts and collapse of organic structures, samples were investigated fully hydrated using an Oxford-Cryosystem attached to the SEM. TEM investigations were carried out in addition (data not shown) with a Zeiss EM 10 instrument.

Data analysis For image and spectra analysis we used PyMCA and Artemis software, a free available software for X-ray fluorescence analysis developed at the European Synchrotron Radiation Facility (Solé et al., 2007). The program allows interactive as well as batch processing of large data sets and is particularly well suited for X-ray imaging. Its implementation of a complete description of the M shell is particularly helpful for analysis of data collected at low energies. The algorithms employed are described in detail elsewhere (Solé et al., 2007). FTIR Data acquisition and processing were performed using Omnic software (version 7.3, Thermo-Nicolet). All measurements on wild type and mutant samples were performed at least three times for equivalent samples or sample spots.

Figure 1 shows the characterization of nerve fibers before, at and after the synchrotron run, in different imaging modalities: optical microscopy (A,B), SEM (D), AFM (E). (A,B) and (E) refer to the same node of Ranvier of a freeze dried specimen which was imaged by x-ray microscopy, as shown in Fig.2, while (D) shows a fiber imaged under cryogenic conditions for comparison. This was made possible by use of a cryo-SEM. For illustration of the functional architecture, a scheme of a node of Ranvier is included in (C). The fiber shown in (A,B,E) was isolated by a teased fiber preparation from sciatic nerve, fixed by rapid cryo-plunging and subsequent freeze drying. It has been deposited on a ultrathin polypropylene foil supported by a gold TEM grid (200 μ m mesh). Compared to samples which were imaged under cryogenic conditions after cryo-plunging (cryo-electron microscopy, see below), without additional freeze drying, a similar overall structure is observed, e.g. see the specimen imaged by FE-SEM microscopy in (D). However, freeze dried samples appear to be flattened. For example, the thickness of the whole fiber in (A,B,D) is in the range of 13.5 μ m in the region of the node of Ranvier and 15.4-17.8 μ m at the paranode. Heights, on the other hand, vary from 1.5 to 3 μ m. Thus, flattening by the freeze drying process and/or by surface interactions must have taken place. With both methods, AFM and SEM, round-shaped features with length scales around 2 μ m and smaller are observed on the fiber surface (Figs. 1D,E). The comparison of AFM and SEM images indicates that this shape of membrane surface structure is preserved after freeze drying.

Samples were routinely inspected before and after the raster-scans in the X-ray microscope without the need of sample transfer into/out-of the chamber. Visible light microscopy images were used to select the node of Ranvier for the x-ray raster scan. Therefore, thick axons with diameters of

approximately 10-15 μm were preferred in the selection process for the x-ray scans.

3. Results

Element mapping in single myelinated axons

Myelinated axons were imaged by synchrotron x-ray fluorescence at different excitation energy. By raster-scanning the sample and mapping the fluorescence yield, the intracellular distribution of the accessible chemical elements can be determined in a single myelinated axon at a spatial resolution corresponding to the beam size, i.e. in the range of 300-800 nm, see above. Here we show x-ray fluorescence maps of representative axons samples of wild type mouse, obtained at 2.9 keV (*FZP-2.9keV* setting, freeze dried preparation), and 7.2 keV photon energy (*KB-7.2keV* setting, frozen hydrated preparation), see Figs.2 and 3, respectively. Some of the samples are also shown in light and AFM microscopy (Fig.1).

Fig.2 shows the absorption and fluorescence map of a freeze dried isolated myelinated axon of a wild type mouse, close to a node of Ranvier, obtained at the FZP-2.9keV setting. The absorption image and summarized fluorescence spectrum is shown in Fig. 2 A and F, respectively. It is possible to clearly distinguish the middle part of the node of Ranvier and the structure of the membranes organized around it. The image was collected on the area of 50 x 25 μm with 246x125 pixels with a dwell time 180 ms per pixel. The fluorescence maps of Na, P, Cl and S are shown in sub-figures (B, C, D, E) respectively. Na and P concentrations show a clear enhancement around the node of Ranvier, by almost a factor of two compared to the internode. The absolute area concentration (given in units of $\mu\text{g}/\text{cm}^2$ in the color bar) of P is 8 fold higher than of Na. On the other hand, Cl and S are distributed quite homogeneously over the whole axon (D, E). The dominating P contribution is not surprising given the high concentration of phospholipids in myelin. The concentrations of the elements vary from 0 to 0.06 $\mu\text{g}/\text{cm}^2$ (corresponding to 3 nmol/ cm^2) for Na, 0 to 21.0 $\mu\text{g}/\text{cm}^2$ (676 nmol/ cm^2) for P, 0 to 0.15 $\mu\text{g}/\text{cm}^2$ (4 nmol/ cm^2) for Cl, and 0 to 0.87 $\mu\text{g}/\text{cm}^2$ (27 nmol/ cm^2) for S.

The results illustrate the potential of x-ray fluorescence imaging to identify element accumulation sites in myelin at the subcellular level. However, the visual results and the above values must be regarded with caution. Firstly, the concentrations are given in units of mass per surface. This is a reasonable unit given that there is no depth resolution in the experiment,

but only as long as the absorption length is either very small or very large with respect to sample thickness, i.e. if thickness variations do not influence the fluorescence yield. For the given photon energy and fiber thickness the absorption length is larger than the fiber thickness. In principle, the area concentrations given above could be converted into volume concentration if the local thickness of the sample was determined, e.g. from the absorption map (Vogt, 2003) or AFM. However, additional assumptions on the stoichiometry and the compound linear attenuation coefficient would be necessary to obtain the averaged volume concentration along the optical path, while a full 3D data set would obviously require a tomographic data set. A second complication arises in the case of the P map, from peak overlap with Os-M lines (e.g. 1.909 keV for Os-M_{α1}, 2.010 keV for P-K_{α1} line), as the sample for the first experiment at 2.9 keV had been stained with Os. The decomposition by full fits allows a distinction based on the lineshape, but the two peaks are not well separated, see Fig.2 (F). Finally, possible changes (artifacts) during the freeze drying process and/or artifacts from Os stain cannot be excluded.

Therefore, further verifications were performed in the next step with the aim to determine the elemental distribution in untreated cryo-fixated (frozen hydrated) samples. The new cryo stage and transfer system at ID 21 offered the opportunity to map the elemental distribution in shock frozen cells under frozen hydrated conditions. Collected spectra of many samples showed very similar spectra, i.e. again the results were reproduced by several samples. For this run, the photon energy was increased to 7.2 keV to cover all elemental lines up to Fe (*KB-7.2keV* setting). Note that the higher flux of the *KB-7.2keV* setting enabled shorter dwell time and thus larger fields of view FOV. Fig.3 (A) shows the summed fluorescence spectrum collected under cryogenic conditions, and (B) a large FOV map, representative of many such results. Parallel oriented nerve fibers deposited on the foil often showed the kind of twisted (helical) structure observable in the image. The fluorescence maps of P, S, Cl, Na, and K are shown in subfigures (C, D, E, F, G) respectively. The strong P, S and Cl contributions in the axons observed in the previous experiment were reproduced. Unfortunately a node of Ranvier was not easily identified in the second run on frozen hydrated samples. The area scanned in (C, D, E, F, G) could eventually show a node of Ranvier. However, we consider it more likely that the map shows a narrowed radius due to the twist of the nerve, as observe in the larger FOV, see Fig.3(A). Due to lack of a fluorescence standard during this run, the fluorescence yield in this case are given in arbitrary units (recorded fluorescence counts). However, the

relative intensities of the fluorescence lines are meaningful. Because of the low concentration and low signal/background ratio, the distribution of Fe, Cu and Mn was more difficult to measure, even though Fe (K-lines), Cu (L-lines) and Mn (K-lines) have been measured, Fig.4(A). Fe was observed usually in form of small patches on the periphery of the cells, without co-localization between Fe, Cu and Mn. For comparison we show the distribution of macroelements P, K and Ca see Fig.4(B) measured at 7.2 keV.

FTIR spectromicroscopy

Fig.5 (A) shows the summed (cumulative) FTIR absorption spectra of a myelinated axon of wild type mouse, as recorded by the SR-FTIR spectromicroscope of ID21. Data was collected on a freeze dried fiber in reflection mode, at the settings described above. A scanning region of interest (ROI) around a node of Ranvier (RN) was chosen for the spectro-microscopy maps, based on the in-line (coaxial) visible light optical microscope (B). An area of $25\ \mu\text{m} \times 80\ \mu\text{m}$ was then mapped with a full high resolution spectrum recorded for each pixel. 2D images can then be generated for all relevant FTIR absorption lines, i.e. in a contrast containing the vibrational information, and thus the fingerprint of the corresponding molecular groups.

In the spectra, the symmetric and antisymmetric CH_2 stretching bands at 2850 and $2925\ \text{cm}^{-1}$, respectively, are most prominent, which is plausible given the high concentration of this moiety in the lipid acyl chains. The corresponding map (C) thus reflect the lipid distribution, clearly showing the gap at the RN. On closer inspection, differences in the distribution of the symmetric and antisymmetric bands are observed, possibly either polarization effects or differences in the corresponding oscillator strength, e.g. due to packing effects.

Towards lower wavenumbers, the next pronounced features in the spectra are the amide I and amide II absorption bands, exhibiting peaks at $1636\ \text{cm}^{-1}$ and $1535\ \text{cm}^{-1}$, respectively, corresponding to vibrations of the protein backbone. The integrated peak intensity of the amide bands can serve as a measure for the local protein concentration. Interestingly, the spatial distribution of these bands is not uniform in the nerve, neither at the RN nor in the compact myelin. Another pronounced peak can be identified as the ester carbonyl stretching band at $1740\ \text{cm}^{-1}$, which may mostly be due to lipids. In order to exploit the fine structure in the amide I region, the fingerprint of the secondary structure of peptides backbones, second derivatives of the spectra were calculated. However, since the peaks are significantly broadened by averaging, modulation of the peaks lineshape are weak and

over-interpretation must be avoided. The overall peak position rather than its fine structure may still be conclusive. The observed peak position of the amide I band at 1636 cm^{-1} is in good agreement with the literature for β -sheet secondary structure at 1633 cm^{-1} , while extended coil and α -helical conformations, are associated with 1646 cm^{-1} , and 1656 cm^{-1} , respectively (Szczerbowska-Boruchowska et al., 2007).

Further absorption bands at 1460 , 1236 and 1057 cm^{-1} were observed. The last two may be associated with the asymmetric and symmetric PO_2^- stretching bands at 1236 and 1086 cm^{-1} , respectively, the first with CH_2 scissor vibration or the CH_3 asymmetric bend vibration (Szczerbowska-Boruchowska et al., 2007). Again caution is required in the interpretation, as long a normal mode analysis and/or extensive reference samples are not exploited.

Diffraction of a single myelinated axon

Fig.6 shows a preliminary single fiber diffraction result on freeze dried nerve fibers, based on the 150 nm 17.5 keV beam (KB optics) at ID22NI. Compared to holography, where the central cone of the diverging beam (indicated as a yellow fan in the schematic) is recorded at large sample-to-detector distance z_2 , the CCD is moved upstream to cover the diffracted signal (blue color in the schematic) outside the central cone of radiation. Note that single fiber diffraction with a beam in the range of $100\text{-}200\text{ nm}$ is a highly challenging task and the setup was far from being fully optimized for small-angle scattering, which is in principle feasible after further instrumental improvements. Here we have used an improvised beamstop to cut the intense direct beam (on the order of 10^{11} cps in the center with two crossed metal bars, which were translated in the beam at a short distance behind the sample. The diffraction signal as measured on the 2D CCD detector is shown in (E), with the shadow of the beamstop appearing, along with the diffraction signal. The diffraction maxima are attributed to the 2^{nd} order lamellar diffraction peaks, and appear as two arcs of enhanced scattering intensity in the upper left and lower right sectors (quadrants) corresponding to the fiber orientation, indicated by the black line. (F) shows a radial slice through the 2^{nd} order lamellar peak (lower left quadrant) reflecting the periodicity of the myelin membranes, plotted against the momentum transfer q , along with a Gaussian fit. As a consequence of the rather oversized and improvised beamstop, the first diffraction order is not fully visible. Only the upper left quadrant shows the 1^{st} peak with the low q tail cut only partially. Least-square fitting of the first and second order results in q values of $0.10\text{ \AA}^{-1}$ and $0.051\text{ \AA}^{-1}$, respectively, with errors in the range of $0.001\text{ \AA}^{-1}$, estimated as

1/10 of the peak width. The corresponding d-spacing of 12.56 nm is plausible for a sample which has undergone shrinkage after freeze drying. We note, however, that this is probably different (as far as we can tell given under the constraints of the beam stop) from the dried state of phase separation of lipid-rich and protein-rich domains Hollingshead et al. (1981). In future, using an optimized setup, the orientation (from the angular peak position), number of membranes (from the peak width) and inter-bilayer spacing (from radial peak position) could be mapped, as the beam is scanned across the node of Ranvier. From this exploratory work, we conclude that single fiber diffraction is possible using a hard x-ray nanoprobe, but the instrument must be further optimized with respect to signal-to-noise, e.g. using proper collimation and beamstop systems. At the high local flux densities, radiation damage at ID22NI was a particular concern. Using hydrated samples in water-Aqua polymount mounting medium (Polysciences Inc., USA) showed very fast and dramatic damages observed in the online optical microscope, with complete loss of the sample in few minutes after measurements. Contrarily, freeze dried samples showed much less observable changes in optical microscopy after irradiation with the nanoprobe beam. Aside from the question whether the water-Aqua polymount mounting medium would leave the structure unaffected, we therefore conclude that cryogenic conditions (vitrified hydrated solution) are needed in order to probe the structure in the hydrated state, as in the fluorescence measurements discussed above. Such frozen hydrated conditions have not yet been tested in nanoscale diffraction.

Soft X-ray tomography

Fig.7 (A) shows a cryo SEM micrograph of a single fiber exhibiting the particular surface structure of a myelinated axon, and (B) shows a transmission X-ray micrograph of a fiber prepared by the same preparation procedure and imaged under the same cryogenic conditions. In contrast to (A) the interior structure becomes visible in absorption contrast, showing an intricate structure at node of Ranvier imaged, which can be well visualized in absorption (density) contrast without staining or slicing. Reconstruction of the 108 images collected at 0.5° intervals through 54° of rotation in the tomographic data set yields the depth resolved structure. A representative slice out of the stack of images is shown in (C). Note that the missing wedge of the data set reduces the achievable 3D resolution. Yet, the preliminary reconstruction outlines the great potential of soft x-ray tomography to unravel the native 3D myelin architecture at the node of Ranvier.

4. Discussion

We have presented a multi-modal synchrotron x-ray microscopy study of individual myelinated axons from wild type mouse. The emphasis was on elemental mappings by x-ray fluorescence, complemented by more exploratory experiments investigating the feasibility and instrumental requirements of single nerve fibers imaging by FTIR spectro-microscopy, nanobeam diffraction, and soft x-ray absorption cryo tomography.

Up to now, only a few in situ studies of elemental distributions in morphologically intact neurons have been performed, mainly by electron microscopy based x-ray microanalysis (EDX) (Rick et al., 1976; LoPachin Jr and Stys, 1995; Lehning et al., 1997; Wroblewski et al., 2000). While electron microscopy requires invasive sample preparation, typically including fixation and thin slicing of the sample, x-ray microscopy can provide spectra without risk of changing the cell organization by chemical fixation or cutting. In particular, the new cryo-stages available on several synchrotron beamlines enable the investigation of cells and tissues after cryo-preservation (plunging in liquid ethane), in the so-called frozen hydrated state without the need of freeze drying procedures. For these conditions, no modification of the oxidation state, and a minimization of beam damage has been reported (Schneider, 1998; Larabell and Le Gros, 2004; Carmona et al., 2008). Another important difference with respect to EDX is related to the depth of the probe. While EDX is surface sensitive to the outer molecular layers, x-ray fluorescence excited by synchrotron radiation collects signal also from within the nerve fiber.

In freeze dried samples we observed a pronounced enrichment of Na and P at the node of Ranvier (RN) of myelinated axons. Unfortunately, it was much more difficult to clearly identify RN regions in the frozen hydrated samples, so that these results could not be verified/falsified for hydrated samples. Na accumulation at the nodes of Ranvier is not surprising, since this part of the fiber contains Na^+/K^+ ATPases, $\text{Na}^+/\text{Ca}^{2+}$ exchangers and high density of voltage-gated Na^+ channels which generate action potentials. However, the high concentration of phosphorus in the node of Ranvier of axons is 1.5 fold higher with respect to the rest of the nerve. P should be present mainly in the myelin sheath due to the contribution of phospholipids, making up about 40 % of the total lipid mass (Siegel, 2006; Quarles et al., 2006). On the other hand, since around node of Ranvier (RN) are numerous plasma membranes, originating from the Schwann cell fingers, as well as the

nodal gap substance, the highly concentrated GM1 gangliosides are likely associated with phospholipids in order to maintain their structural incorporation into membranes. Further, the enhanced phosphorus concentration at the RN indicates the presence possibly high energy metabolites such as ATP. Receptors for extracellular ATP have been discussed in view of sensory transduction (Burnstock and Williams, 2000). ATP can also act as an activity-depend signalling molecule in communication between neurons and glia (Fields and Stevens, 2000). Furthermore, mitochondria necessarily for ATP production are localized mostly around the RN (Edgar et al., 2008). Another contribution to P accumulation may stem from cyclic AMP (cAMP). Myelin formation by oligodendrocytes has been shown to be regulated by cAMP signaling pathways and to depend on the resting membrane potential (Bolton and Butt, 2006). In an early EDX study, Rick and co-workers have observed high Na concentration in the axon near the node of Ranvier, but did not comment on P, which also was found to be high in comparison to surrounding tissue (Rick et al., 1976). The authors concluded that P was connected only to phospholipids.

From FTIR spectromicroscopy, information on protein secondary structure can be inferred, averaging locally over all proteins in the excitation volume. Proteins make up 30% of the dry weight of myelin. The most abundant PNS myelin proteins are protein zero (P0), basic protein (MBP) and myelin-associated glycoprotein (MAG). P0, a transmembrane glycoprotein like MA, belongs to the immunoglobulin gene superfamily (Lemke et al., 1988). Here we explain the observed peak positions of the amide bands by predominantly β -sheet conformations. The spatial distribution of the proteins is distinctly different from the lipids, but also peaks in the region of compact myelin, rather than at the RN.

We have also shown that the multilamellar ordering of the myelin sheath can be addressed by diffraction with nanometer-sized hard x-ray beams, a relatively new technique which could significantly extend the capabilities of classical small-angle X-ray scattering studies (Fratzl, 2003; Aichmayer et al., 2005), which average over large ensembles. However, we were not able yet to fully exploit this potential due to technical difficulties, since strong focusing and diffraction are not easily made compatible and will require a more dedicated instrumentation (beamstop, optical elements for background reduction) to 'clean up' the diffraction pattern. However, the signal level was found to be sufficient for scanning diffraction microscopy. Another concern is radiation damage, since the flux density is particularly high at the nanoprobe

beamline, and systematic investigations of the effect in varying accumulation time should be performed in the next step. After future optimization, we expect that simultaneous determination of the lamellar periodicity d (from radial peak position), membrane orientation (from the angular peak position), and possibly the number of adjacent membranes N (from the peak width) can be mapped. Highly resolved images of these quantities in interesting regions, e.g. around the node of Ranvier could help to unravel the evolution of the structural organization and the associated pathologies. A further advantage of the nano-focus hard x-ray probe derives from the fact that different imaging modalities can be combined, in particular x-ray fluorescence, holography (shown but not exploited here) and diffraction. A first step towards this direction has been presented here. Furthermore, a leadoff experiment on soft x-ray tomography has shown that single fiber tomography under cryogenic conditions has now become possible with the new BESSY x-ray microscope. In contrast to electron tomography, which still is superior in resolution for thin samples (Sosinsky et al., 2005; Carrascosa et al., 2009), sectioning and staining is not required.

Finally, the multi-modal x-ray imaging approach presented here may also be used in future to study pathological processes involved in myelin related neurodegenerative diseases, such as multiple sclerosis or the tomaculous neuropathy following hypermyelination. Understanding of the responsible mechanisms requires methods that assess both spatially resolved morphological and chemical alterations in myelin.

In summary, recent progress in synchrotron x-ray optics, enables raster-scanning of individual freeze dried or frozen hydrated nerve fibers to assess (i) the chemical composition from x-ray fluorescence, (ii) the projected electron density from absorption/phase shift measurements, and (iii) the local spacing and orientation from diffraction, respectively, all with sub-micron resolution.

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Figure Legends

Figure 1.

(A,B) Visible light microscopy images of an isolated teased fiber preparation from sciatic nerve, fixed by rapid cryo-plunging and subsequent freeze drying: (A) as observed at the beamline (Zeiss Axioplan 2, 100x Epiplan-NEOFLUAR 100/0.90, DIC) before mounting the sample for the x-ray spectro-microscopy experiment, and (B) as observed in the in-line optical microscope after sample transfer into the x-ray microscope chamber. The red circle indicates a node of Ranvier selected for the x-ray raster scan. (C) Schematic of the myelinated axon with node of Ranvier and paranodal loops. (D) FE-SEM micrograph of cryo-fixated myelin fibers (LEO 1530 Gemini, 1kV). (E) AFM microscopy (tapping mode amplitude, $35\mu\text{m} \times 35\mu\text{m}$ scan size) of a freeze dried fiber around node of Ranvier, recorded after the synchrotron experiment. (A,B,E) are images of the same fiber which is also shown in Fig.2 (below), plunge frozen and freeze dried on the polypropylene membrane.

Figure 2.

(A) Absorption x-ray image of myelinated axon obtained at 2.9 keV photon energy, (B) Fluorescence image of sodium, (C) chloride, (D) phosphorous and (E) sulphur in the single isolated axon shown previously in light and AFM mode, in Figs. 1A, B and 2, respectively. Scales indicate elements concentration in $\mu\text{g}/\text{cm}^2$. Image size $50 \times 25\mu\text{m}$, 246×125 pixels with dwell time 180 ms per pixel. (F) The summed fluorescence spectrum of the scanned area along with a fit (sum: black line, fit: dark red line, individual peaks: see legend).

Figure 3.

X-ray fluorescence maps similar to Fig.2, but for a frozen hydrated preparation of nerve fibers imaged at cryogenic conditions, and at higher photon energy of 7.2 keV. (A) Cumulative fluorescence spectrum with least-square fit (sum: solid black line, individual peaks: Na-yellow, Si-gray, P-red, S-light blue, Cl-violet, K-dark blue, Ca-green, Mn-pink, Fe-orange and Compton scattering-gray). (B) An overview scan over an area of $100 \times 100\mu\text{m}$, showing the P fluorescence of parallel and partly twisted nerve fibers. (C,D,E,F,K) Close-up scans around an area of $20 \times 15\mu\text{m}$, showing the P, S, Cl, Na and K fluorescence distribution, respectively. The pixel size corresponds to $0.3 \times 0.3\mu\text{m}$.

Figure 4.

X-ray fluorescence of cryo-preserved myelinated neuron. Overlaying distribution of microelements (Mn (red), Fe (blue) and Cu (green) in situ (A) and the distribution of macroelements: P (red), Cl (green) and K (blue) (B) in the single isolated neuron. Image size $70 \times 40 \mu\text{m}$, 280×160 pixels with dwell time 150 ms per pixel. $E=7.2 \text{ keV}$.

Figure 5.

(A) The summed (cumulative) FTIR absorption spectra of a myelinated axon of wild type mouse, as recorded in reflection mode by the SR-FTIR spectromicroscope of ID21. Data were collected on a freeze dried fiber in reflection mode, at the settings described above. A scanning region of interest (ROI) around a node of Ranvier (RN) was chosen for the spectro-microscopy maps, based on the in-line (coaxial) visible light optical microscope (B). An area of $25 \mu\text{m} \times 80 \mu\text{m}$ was then mapped with a full high resolution spectra recorded in each pixel. 2D images can then be generated for all relevant FTIR absorption lines, i.e. in a contrast containing the vibrational information, and thus the fingerprint of the corresponding molecular groups.

Figure 6.

(A) Schematic of the hard (17.5 keV) x-ray nanoprobe experiment at beamline ID22NI with focusing mirrors, sample stage, CCD farfield detector, and fluorescence detector. The myelinated axons were deposited on the polypropylene foil supported by an orientation grid, and could be scanned by piezo motors through the 150nm beam. The fibers were adjusted, controlled and imaged by three independent imaging modalities: light microscopy as shown in (B), inline holography (C) recorded with a farfield CCD detector while the sample is moved out of focus by small defocus distances z_1 , fluorescence as shown in (D). The fluorescence map shows the distribution of the Zn- $K\alpha$ map, which was not accessible in the ID21 experiment at lower excitation energy, covering an image area $30 \mu\text{m} \times 10 \mu\text{m}$ with 150×50 pixels and 100ms dwell time per pixel at 17.5 keV. The diffraction signal as measured on the 2D CCD detector is shown in (E). Compared to holography, where the central cone of the diverging beam (indicated as a yellow fan in the schematic) is recorded at large sample-to-detector distance z_2 , the CCD is moved upstream to cover the diffracted signal (blue color in the schematic) outside the central cone of radiation. The cross pattern visible in (E) reflects

the absorption of a beamstop behind the sample, while the two arcs of enhanced scattering intensity in the upper left and lower right sectors reflect the lamellar scattering of a single fiber oriented along the direction of the black line. The diffraction maxima are attributed to the 2nd order lamellar diffraction peaks, and appear as two arcs of enhanced scattering intensity in the upper left and lower right sectors (quadrants) corresponding to the fiber orientation, indicated by the black line. (F) shows a radial slice through the 2nd order lamellar peak (lower left quadrant) reflecting the periodicity of the myelin membranes, plotted against the momentum transfer q , along with a Gaussian fit. As a consequence of the rather oversized and improvised beamstop, the first diffraction order is not fully visible. Only the upper left quadrant shows the 1st peak with the low q tail cut only partially. Least-square fitting of the first and second order results in q values of 0.10 and 0.051 Å⁻¹, respectively, with errors in the range of 0.001 Å⁻¹, estimated as 1/10 of the peak width. The corresponding d-spacing of 12.56 nm is plausible for a sample which has undergone shrinkage after freeze drying.

Figure 7.

(A) Cryo SEM micrograph of a single fiber exhibiting the particular surface structure. (B) Transmission X-ray micrograph of a fiber prepared and imaged under the same cryogenic conditions. The interior membrane organization near a node of Ranvier is visualized by a projection image in absorption contrast (transmitted intensity in linear grey scale). (C) Representative reconstruction slice from a tilt series showing the absorption coefficient.

Figure 1:

Figure 2:

Figure 3:

Figure 4:

Figure 5:

Figure 6:

Figure 7: