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A scanning SAXS/WAXS study of rat brain

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Abstract. A simultaneous SAXS (small-angle X-ray scattering) and WAXS (wide-angle X-ray scattering) measurement setup was installed at BL45XU in SPring-8. The system comprises of a short (specimen-to-sample distance about 50cm) vacuum path and a mosaic CCD detector. It covers a q-range of 0.02-2.5 nm⁻¹. Using this setup, lipids in formalin-fixed rat brain were analyzed. A brain slice was moved across the X-ray beam with a step size of 0.5 mm to map reflections from lipids in various areas of brain. White matter that contains myelin gave strong lamellar reflections in the small-angle region which are often anisotropic. Gray matter shows only a central scatter in the small-angle region. In the wide angle region, both white and gray matters gave rise to sharp rings that are due to lateral packing of hydrocarbon chains in the lipid membranes. The relative intensities of these rings were different in white and gray matters, showing that the lateral arrangements of the lipids in bilayers are different.

1. Introduction
Most of biological tissues have a hierarchical structure that comprises of distinct units at different levels of scale. Since the structures at different scales give X-ray diffraction or scattering in different angular ranges, it is often useful to record the small- and wide-angle diffraction from the same sample to understand its structure. At synchrotron radiation facilities, this has been realized by using separate detectors for the small- and wide-angle regions [1,2].

Brain is a typical example of a hierarchical structure. Brain is mostly made of neuronal cells that are rich in lipids. Different regions of brain are specified by their appearance (color) such as white, gray and dark matters. The major reason for the different appearances is a difference in the lipid structure and composition. Gray matter is mostly made of cell bodies and neurons while white matter contains axons that are shielded by myelin. Myelin is produced by Schwann cells and structurally a stack of double bilayers of lipids. Myelin appears white, providing the origin of the color of white matter. The chemical compositions and the structure of lipids in brain are strongly related to its medical conditions. It has been reported that lipid modifications in gliomas, the most frequent primary brain tumors, correlate with their malignancy [3]. It has been also shown that, unlike normal myelin, multiple sclerosis myelin does not show a 4.15nm peak that represents gel phase, possibly as a result of different protein-lipid interaction [4]. Myelin in brain tumors has been studied by X-ray diffraction recently [5-7], but the functional relation between its lipid constituents and brain activity is not well understood.

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In the present study, slices of rat cerebrum were subjected to a scanning SAXS/WAXS measurement to study the lipid structure. The experimental setup is based on the use of a large CCD detector for protein crystallography. This technique allows us to make a two-dimensional distribution map of materials in a sample. This is extremely useful for a specimen like brain in which different constituents are mixed but segregated in some regions.

2. Materials and Methods

2.1. Preparation of brain samples

Cerebrum was excised from male/female Wister or Sprague-Dawley rats that were used in experiments at SPring-8 (mostly for the studies on cardiac muscle). After the experiments, the rats were killed by an overdose of pentobarbital and the brains were dissected out. They were put into neutral-buffered 10% formalin and kept in a refrigerator for one week to three months. No difference was observed with the preservation period nor when the sample was preserved at room temperature. The formalin-fixed brains were sliced with a razor to a thickness of 1-2 mm for 2D scanning. The slices were kept in a phosphate-buffered saline (pH 7.0) and sealed between two sheets of Mylar (thickness 6 µm) with the saline. X-ray measurements were made at room temperature.

2.2. Simultaneous WAXRD and SAXS measurement at BL45XU

BL45XU has two tandem helical undulators as an X-ray source and uses a diamond crystal as a beam splitter [8,9]. In the present experiment, the protein crystallography hutch with three sets of diamond double-crystal monochromators was used. One set of the monochromators was tuned to obtain a 13.8 keV X-ray, while the others were moved out of the beam. A bent cylindrical mirror was used to focus the X-ray beam. The beam size was approximately 250 µm × 250 µm at the sample. The sample-to-detector distance was 450 mm. At 15 mm upstream to the specimen was a guard pinhole with a diameter of 0.8 mm (Figure 1a). The flux was about 2×10¹¹ cps. The X-ray detector was Jupiter210 (RIGAKU, Tokyo, Japan), which comprises of four CCDs with tapered fibers. The detection area was 205 mm × 205 mm. The pixel size was 100 µm × 100 µm with 2×2 binning. In order to cover a wide q-range, the detector was set at an offset position so that the beam stop was near the bottom of the detection area (Figure 1b). The SAXS and WAXS regions were measured simultaneously. A fiber diffraction pattern from dried chicken tendon collagen (Figure 1c) shows the 3rd-order meridional reflection at a Bragg spacing of about 1/21 nm⁻¹ and the meridional reflection at 1/0.28 nm⁻¹ (Figure 1b), demonstrating that this setup covers a q-range of about two orders of magnitude (q=4πsin(θ)/λ, the Bragg spacing d=2π/λ, where 2θ is the scattering angle and λ is the wavelength). Since the SAXS intensity exceeds the WAXS intensity by nearly three orders of magnitude and the dynamic range of the detector was several thousand, the software for the measurement control was designed to allow multiple exposures with different exposure time. Thus, to measure the wide-angle region with good statistics and to avoid saturation in the small-angle region, images with three different exposure times (typically 2, 5 and 30 sec) were recorded from the same spot of the sample. The dark images (images taken with the same exposure time without X-rays) was subtracted from each image. Also, a small (<5%) correction was made to cancel the differences in amplification of the four CCDs. For intensity measurements, a diffraction pattern was averaged circularly to obtain a one-dimensional intensity profile. Then, the integrated intensities of peaks were obtained by fitting a polynomial (usually a second order) background and Gaussian peaks whose center positions were used as the peak positions.

3. Results

3.1 Small-angle X-ray scattering (SAXS)

The SAXS patterns from rat brain differ markedly in regions. In gray matter of cerebrum that includes cerebral cortex and caudate nuclei, only diffuse scatter was observed in the small-angle region. On the other hand, in white matter including external capsule, strong rings or arcs were observed showing the abundance of myelin sheaths (Figure 2a). These can be indexed on a fundamental repeat of 14.4 nm.
This is shorter than the repeat distance in unfixed brain (15.9 nm) and other reports (18.0 nm in native rabbit sciatic nerve, 15.6 nm in rabbit optic nerve, 17.0 nm in frog sciatic nerve myelin [10]. Most probably, formalin reduced the distance between lipid bilayers by cross-linking the proteins that are located outside of the bilayer. From formalin-fixed human cerebral myelin, De Felici et al.[5] observed a 16.0-16.6nm periodicity, but the length of fixation was only a few days in their experiments while it was longer than one week in the present case. The myelin reflections were usually observed up to the sixth order and often showed orientation indicating the direction of the axons. There were also other weak rings of unknown origin such as broad maxima at d=1.1 and 0.72 nm. As for the former, Inouye and Kirschner [11] observed a weak diffuse ring at d=1.1 nm in central myelinated tissue which they attributed to myelin protein.

3.2 Wide angle X-ray diffraction (WAXD)

The WAXD patterns from formalin-fixed brain samples showed well-defined peaks in both gray and white matters (Figure 2b), which can be attributed to lateral packing of lipid molecules in bilayers. There were several peaks (Figure 3): a weak, broad peak at q=14.26 nm$^{-1}$, a weak, sharp peak at 14.68 nm$^{-1}$, a small shoulder at 14.81 nm$^{-1}$, a well-defined strong peak at 14.93 nm$^{-1}$, another well-defined strong peak at 15.27 nm$^{-1}$, a broad, weak shoulder at 15.48 nm$^{-1}$ and a broad peak at 16.57 nm$^{-1}$. There were other rings that were not observed constantly. Slices of brains not fixed with formalin did not show sharp peaks in white or gray matter. Occasionally, a broad peak was observed around q=15 nm$^{-1}$. The intensity of the peaks varied according to the areas of brain. Especially, the relative intensity of the two strong reflections at 14.93 nm$^{-1}$ (d=0.421nm) and 15.27 nm$^{-1}$ (d=0.411nm) reversed between

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**Figure 1.** The SAXS/WAXD measurement setup at the BL45XU protein crystallography hutch in SPring-8. (a) Arrangement in the experimental hutch. The area detector (Jupiter210, RIGAKU, Tokyo, Japan) was placed off-center to cover a large q-range. (b) A wide-angle diffraction pattern from collagen (dried chicken tendon). The strong arc at the top is the 0.28nm meridional reflection. The black lines are unusable regions due to gaps between tapered fibers and to boundary between segments of the CCDs. (c) A small-angle diffraction pattern from collagen. The peak just outside of the beam stop (pointed by an arrow) is the third order meridional reflection from the 63nm periodicity.
white and gray matters (Figure 3). From formalin-fixed human cerebral myelin, De Felici et al. [5] observed a peak at \(q=14.4 \text{ nm}^{-1}\) (\(d=0.436\text{nm}\)) that may correspond to the former peak but much broader than in the current experiment.

3.3 2D mapping of the SAXS and WAXD

To investigate the relative intensity of the lipid-derived peaks, diffraction patterns were obtained from different regions of cerebrum by moving the sample with 0.5mm steps in two dimensions. Figure 4 shows maps of intensity of the myelin 4th-order reflection and peaks at 14.93 and 15.27 \text{nm}^{-1}. The myelin reflection was generally strongest in white matter. Since the slice is 1-2mm thick, there may be white matter that cannot be seen in the photograph shown in Figure 4a. This may have somewhat blurred the map. The distribution of intensity of the myelin reflection (Figure 4b) is similar to that of the wide-angle reflection at 14.93 \text{nm}^{-1} (\(d=0.421\text{nm}\)) (Figure 4c). On the other hand, the reflection at 15.27 \text{nm}^{-1} (\(d=0.411\text{nm}\)) is stronger in the cortex and the central part of cerebrum (Figure 4d). Although the two distributions are not mutually exclusive, it is clear that white and dark matters are characterized by these reflections. The distribution of the intensity of the reflections at 14.68 \text{nm}^{-1} and 16.57 \text{nm}^{-1} also correlated weakly with that of the myelin reflection.

Figure 2. The SAXS/WAXD recording from formalin-fixed white matter (myelin) of rat cerebrum. (a) Small-angle region recorded with an exposure time of 2 sec, showing the strong second order peak from myelin. Although not apparent in this pattern, there were higher orders which were often oriented and appeared as arcs. (b) Wide-angle region recorded with a 30sec exposure showing sharp rings from lipids and a broad ring from water. The small-angle region is saturated. In these images, after dark subtraction, the gaps between the tapered fibers and other defects were filled by interpolation.

Figure 3. The wide-angle intensity profiles observed from rat cerebrum. Profiles obtained in two regions, white and gray matters, are shown. The high background is from other components in the specimen such as proteins and water.
4. Discussion

It is not clear why only formalin-fixed brain gives sharp rings due to the lateral packing of lipids. Since brain lipid bilayers have a considerable amount of protein, fixation of proteins, especially cross-linking of side chains of lysine and arginine, is conceivable. On the other hand, as there has been reports that formaldehyde combines with unsaturated lipids at the double bond [12,13], effects on lipid molecules are also possible. Since there has not been a report showing sharp lipid peaks before, the extended fixation used in this study may be effective in inducing lateral orders of lipid molecules.

There is a marked difference in the lipid composition between gray and white matters. In gray matter, glycerophosphatides are dominant with some sphingolipids and cholesterol (ratio 21:5:7 in dry weight percent in adult human brain [14]), while in white matter there are much more sphingolipids and

![Figure 4. An example of scanning results of a coronal section of rat cerebrum. (a) A micrograph of the specimen. The area boxed by the red line was scanned. W denotes white matter, G gray matter. (b) The distribution of intensity of the 4th-order reflection from myelin (at 3.6 nm). The sample was scanned from the top left to the right bottom, with 27 steps horizontally and 19 steps vertically. The size of each step was 0.5 mm. (c) The distribution of intensity of the lipid peak at d=0.421 nm. (d) Distribution of the intensity of the lipid peak at d=0.411 nm. The yellow dots in (c) and (d) are where the diffraction patterns of myelin and gray matter in Figure 3 were obtained.](image-url)
cholesterol with the ratio of 21:21:15. This is mainly due to the composition in myelin which has a ratio of 24:24:19.

It has been found that the presence of cholesterol in DPPC (dipalmitoylphosphatidylcholine) / DOPC (dioleoylphosphatidylcholine) model membrane system causes disorder in chain-packing and increases the lateral distance between lipid chains [15]. It was reported that with DPPC:DOPC=1:1 the X-ray peak due to lateral packing was at d=0.415 nm while with DPPC:DOPC:cholesterol=1:1:0.5 (molar ratio), the peak was at 0.428 nm. Thus, the higher cholesterol content may be the reason the 0.421nm peak is stronger than the 0.411nm peak in myelin in the present study.

This is the first study to investigate lipid structures in different regions of brain and preliminary in nature. It is possible that only a small fraction of lipids in these regions is contributing to the sharp peaks. Presence of other reflections show that there are other types of components in brain. Since brain is a very complicated organ, there must be different lipids playing different roles. Especially in gray matter, lipids may be not only in cellular membranes that transmit electrical signals but also in subcellular components. Information on the structure and composition of lipids in the subcellular organs is scarce. However, the structural difference between the lipids in gray and white matters shows that there are fundamental variations which may be related to the functions of lipids in these regions.

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References