Mineralized Microstructure of Calcified Avian Tendons: A Scanning Small Angle X-ray Scattering Study

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Abstract. The micrometer level spatial distribution of the size, shape, and orientation of mineral crystallites in the calcifying matrix of tendons near the edge of the mineralizing front was investigated by scanning small angle X-ray scattering using synchrotron X-ray radiation. Using a special microbeam arrangement enabling 20 μm beam resolution and short measurement times, linear diffraction scans were made on sections from the normally calcifying tendons (tibialis cranialis) from the domestic turkey, which calcify in the distal to proximal direction. A change in shape and arrangement of mineral crystals was observed within the first 200 μm of the mineralization front, and the mineral crystal distribution was highly anisotropic with crystals aligned parallel to the fiber axis. In a cross-section of the tendon cut at right angles to the fiber axis, the orientation distribution of crystals was not azimuthally symmetric, and showed a small but nonzero anisotropy and a continuous change in mean orientation angle across the width of the tendon cross-section.

Key words: Apatite crystal — Biomineralization — Small angle X-ray scattering — Synchrotron radiation — Turkey tendon

The early stages of mineralization in bone and related tissues like the mineralizing turkey leg tendon (MTLT) have been investigated extensively to determine the growth and development of the mineral crystals in the collagen matrix (mineralized collagen nanostructure). Techniques such as transmission electron microscopy (TEM) [1], three-dimensional tomography from high voltage TEM [2], and atomic force microscopy (AFM) [3] have been used to investigate the fibril length scale (of the order of tens of nanometers), and have shown that the initial mineral deposits are found in matrix vesicles outside of the collagen fibrils [4], the mineral particles are plate-like in appearance and can grow by coplanar fusion [2], and that within the fibrils, the mineral deposits first appear in the gap zone [5].

However, a large gap in the observed length scales exists between the 10–100 nm regions observed in electron microscopy and the macroscopic (millimeters to meters) level. Indeed, many of the particular features of a calcified tissue, such as its anisotropic mechanical properties, depend on the arrangement of fibrils and groups of fibrils into arrays and higher ordered structures between 100 nm and 0.1 mm [6]. In addition, the size and shape of the mineral crystals and their interrelation to the collagen matrix can vary spatially over the length scale of 1–100 μm, either in the natural state — for example, across the bone-cartilage interface [7] — or in bones of patients with bone or bone-affecting disorders like coeliac or Paget’s disease [8]. As an example, in dentin the gradations in the material properties at the micron length scale (size, shape, and density of mineral crystals) have been found to correlate with the changes in material properties near the dentin-enamel junction, improving the resistance to crack propagation [9]. Thus, both the micron level change in tissue architecture, as well as the accompanying changes in the typical nanostructure are essential to develop a complete picture of the physiological development of calcified tissues.

Clearly, investigation of such changes of material properties at this micron length scale is sufficiently time consuming and data intensive as to be impractical with nanometer level probes like electron microscopy. Considerable effort has therefore been devoted to the development of methods that probe structural variation on a larger length scale, including but not limited to micro-tomography (μCT) and magnetic resonance microimaging (MR μI) for the structural parameters of bone at the tissue level [10], Fourier transform infra-red microspectroscopy (μ-FTIR) which gives information on collagen cross-linking, carbonate:phosphate and mineral: matrix ratios [11, 12], wide-angle X-ray diffraction which measures the
crystallographic orientation of the mineral particles and degree of crystallinity [13, 14], and small-angle X-ray scattering, which provides information on the shape, size, and orientation of the mineral particles [15, 31].

Scanning small-angle X-ray scattering (sSAXS) is a nondestructive method of investigating mineralized tissues which combines information at two length scales — micron and nanometer — to obtain a micron level map of the nanometer level material properties of bone. More precisely, small angle X-ray scattering (SAXS) of materials containing particles in the nanometer range reveals information on the average size, shape, and orientation of the particles in the volume irradiated by the incident X-ray beam [15, 16], and has been used successfully to investigate the structure of mineral crystals in bone [15, 32] and of cellulose fibrils in wood [17, 18], for example.

Therefore, by using a X-ray probe beam with a given diameter \( d \) in the micron range, which can be positioned with \( \mu \)m-level accuracy over the sample, one can obtain 1D or 2D parameter maps of the mineralized nanostructure (nanometer level) with position resolution \( d \) (micron level). Using a conventional laboratory source with cross-coupled Göbel mirrors and a gas proportional counter area detector [19], the beam diameter \( d \) needs to be at least 100–200 \( \mu \)m in order to have enough incident X-ray photons to measure a single point in the sample with sufficient statistics in a reasonable period of time (1/2–1 hour).

In order to increase both position resolution and decrease time for data acquisition, the high brilliance of X-ray radiation obtainable from synchrotron sources is necessary. Recently, we have developed a setup for sSAXS measurements at synchrotron beamlines, with which we can obtain beam diameters of as low as 10 \( \mu \)m (1/1000th of the irradiated volume using a laboratory source), and short sample measurement times of 180–300 seconds/point (factor of 10–20 improvement) [7].

It is the aim of this paper to demonstrate the utility of the microbeam scanning SAXS technique and its high position resolution by applying it to the mineralizing turkey leg tendon (MTLT). This system has been extensively investigated at the nanometer level by electron microscopy because of its use as a model for the early stages of vertebrate calcification [1, 5, 20], with a parallel fibered architecture and progressive mineralization in distal to proximal direction. However, not much is known about the mineral distribution and spatial variation of mineral crystal parameters on a length scale from 1 to 100 \( \mu \)m. In this work we investigate the changes in mineral nanostructure in MTLT over length scales of up to 200–500 \( \mu \)m both parallel and transverse to the fiber axis near the edge of mineralization, with 20 \( \mu \)m position resolution.

### Materials and Methods

#### Sample Preparation

Partially mineralized tibialis cranialis tendons were dissected from the legs of a male turkey sacrificed at 23 weeks of age, and frozen at approximately \(-18\)°C. Subsequently, the tendons were thawed in phosphate-buffered saline (PBS) at 4°C, and microradiography measurements (ISO-Debyeflex 1001, Seifert, Hamburg, Germany) were carried out. Tendons were dehydrated in a graded series of ethanol solutions, and embedded in polymethylmethacrylate (PMMA) [21]. Sections 30–50 \( \mu \)m thick were cut parallel (longitudinal) and perpendicular (transverse) to the fiber axis. Longitudinal sections included the transition from mineralized to unmineralized collagen, and transverse sections were about 6 mm distal from this transition.

#### Scanning Electron Microscopy (SEM)

Samples were coated with a vacuum-deposited layer of carbon and investigated in a digital scanning electron microscope (SEM) with a four quadrant semiconductor back-scattered electron (BE) detector (DSM 962, Zeiss, Oberkochen, Germany) at a sample-lens distance of 15 mm, with probe current \( 110 \pm 0.4 \) pA and electron beam energy 20 keV. In the resulting gray-level image of the mineralized tissue, lighter regions denote regions with higher mineral concentration. A mean mineral weight and volume percentage in the imaged region of the calcified tissue, excluding unmineralized tissue, cracks, and embedding material, can be obtained from the gray-level image (for details see [21]). Samples were photographed in a polarized light microscope, enclosed in lead masks to expose only a selected region of interest.

#### Scanning Small-Angle X-ray Scattering (sSAXS)

sSAXS measurements were carried out at the SAXS beamline (Austrian Academy of Sciences) at Sincrotrone Trieste, Italy, a third generation synchrotron radiation source. The basic idea behind the method is to combine a 2-pinhole system (20 \( \mu \)m and 100 \( \mu \)m) with the high brilliance of synchrotron radiation to obtain a 20 \( \mu \)m X-ray beam with high photon flux \( (5 \times 10^8 \) photons/second, wavelength \( \lambda = 0.154 \text{nm} \)). By combining the small beam diameter with a sample which can be translated in the plane perpendicular to the beam with an accuracy of better than 1 \( \mu \)m, the nanostructure of the tissue can be investigated over volumes of the order of (10 \( \mu \)m)

The principal components of the experimental apparatus are shown in Figure 1(A). Circular Pt/Ir pinholes (Christine Gröpl Elektronenmikroskopie, Tulln, Austria) were mounted on a pinhole alignment stage and aligned by means of a step-motor controlling unit. The sample holder was mounted on vertical and horizontal translation stages (linear encoder motors connected to a Newport controller) allowing 250 mm horizontal and 9 mm vertical travel distance, and better than 1 \( \mu \)m positioning accuracy. Immediately following the sample, a diode (PIN-diode, IRD, Torrance, CA, USA) may be inserted for measurement of the intensity of the X-ray beam transmitted through the sample. To minimize air scattering, the remainder of the path to the detector was through an evacuated tube with Mylar windows. A 2D position-sensitive CCD detector (Bruker AXS, Karlsruhe, Germany) was located at the end of the evacuated tube to measure the SAXS pattern (512 \times 512 pixels and 64 mm \times 64 mm active area). Sample to detector distance was 438 mm. Reflection of the direct beam from the left window of the evacuated tube was read by a line detector positioned above the beam path to correct for variations in the primary beam intensity. All alignment, sample positioning, and measurement protocols were carried out with control software designed in a modular arrangement [22].