L12

REVEALING THE NANOSTRUCTURE OF BIOLOGICAL MATERIALS USING SCANNING X-RAY IMAGING WITH SAXS CONTRAST

A. Gourrier [1,2*], C. Riekel [2], and J. Doucet [1]

[1] *Laboratoire de Physique des Solides, Bat. 510, Université Paris-Sud, 91405 Orsay Cedex, France*
[2] *ESRF, 6 rue Jules Horowitz, 38043 Grenoble cedex, France*

Keywords: biological materials, biomineralization, nanostructure, X-ray imaging, SAXS, microbeam

**) e-mail: gourrier@lps.u-psud.fr*

Biological materials often exhibit complex structures generally extending over several length scales. This essentially results from the optimization of the growth processes to attain the desired function within a given set of restricted environmental conditions [1, 2]. Despite of the great variety of structures found in nature, at the fundamental level, hard materials usually show common patterns of organic macromolecules embedded in a mineral phase. Thus, the most basic events in biomineralization occur at the nanometer level through self-assembly processes [3]. More recently, it was recognized that natural (and synthetic) composites with nanometer-sized features (particles, layers...) could achieve exceptional properties [4]. These observations are therefore currently driving a widespread effort in the direction of a better understanding of the structure of biomineralized materials at the nanoscale.

Due to the heterogeneous nature of the mineralized tissues, the difficulty of a precise characterization lies in the necessity to correlate the shape and organization of the nanoscale features with the microstructure. Typical film/particle thicknesses in, *e.g.* shells, bones and teeth, are in the order of 1-10 nm arranged in larger structures of up to tens of mm^2 in cross-section. In most cases several techniques therefore need to be combined since there is always a trade-off between the resolution that is needed to measure the nanoscopic heterogeneities and the field of view required to image the microstructure.

Synchrotron facilities have provided extremely powerful tools to address such questions. In this respect, small- and wide-angle X-ray scattering (SAXS/WAXS) experiments now constitute one of the major classes of synchrotron experiments in biology. This technique allows bridging the gap between the information obtained at the atomic/molecular level by macromolecular crystallography or spectroscopy and this of the cells or tissues by imaging. The growing impact of SAXS/WAXS methods essentially results from the increase in brightness by orders of magnitude in second and third-generation sources as the ESRF as compared to conventional X-ray laboratory equipment. The first benefit of using such sources comes from the considerable increase in data collection rate which ultimately allows real-time studies.

A second important development stems from the recent advances in X-ray optics which have paved the way for position-resolved measurements [5]. Scanning SAXS/WAXS therefore allows mapping structural parameters related to the atomic and molecular order as well as average shape, size and orientation of nanometre-sized heterogeneities within the region probed by the beam. This enables the reconstruction of images where each pixel is a representation of the local value of a nanostructural parameter obtained from the analysis of the scattering pattern. The lateral resolution of this image is thus given by the beam size in first approximation. However, this method relies on a systematic analysis of the scattering patterns, which becomes rather tedious as the number of frames increases by orders of magnitude due to smaller beam sizes and faster detector read-out time, allowing larger areas to be covered.

This work intends to demonstrate how this technique could be further developed in the direction of more standard full-field X-ray imaging techniques using small angle scattering as source of contrast.

The first example chosen to illustrate this technique is this of the eggshell (Fig. 1), a model for biomineralization studies. Eggshell is a composite that forms by deposition of calcite on the inner protein membrane which further directs the overall growth of the shell [7]. At present, the intimate relationship between the original protein matrix and the mineralizing phase is still poorly understood. In this study, a region of interest on the mammillary layer of the eggshell section was selected by optical microscopy (box in Fig. 1a). This layer is responsible for the initial spherulitic growth processes.

A sample section was scanned at the ID13 beamline of the ESRF using a focussed beam of $1\times1\mu m^2$ over an area of 130×100 μm^2 ($h\times v$) in steps of 2×5 μm^2 ($h\times v$). The transmitted intensity shown in Fig. 1b was measured at each scan point using a photodiode. It appears higher in the mammillary membrane and darker in the mineralized part, *i.e.* regions II and III respectively. This information is qualitatively equivalent to a classical radiography of the sample where the contrast is due to a higher X-ray absorption by the mineral phase. Note that the decrease in intensity in region I of the image is due to absorption by the embedding resin, PMMA.

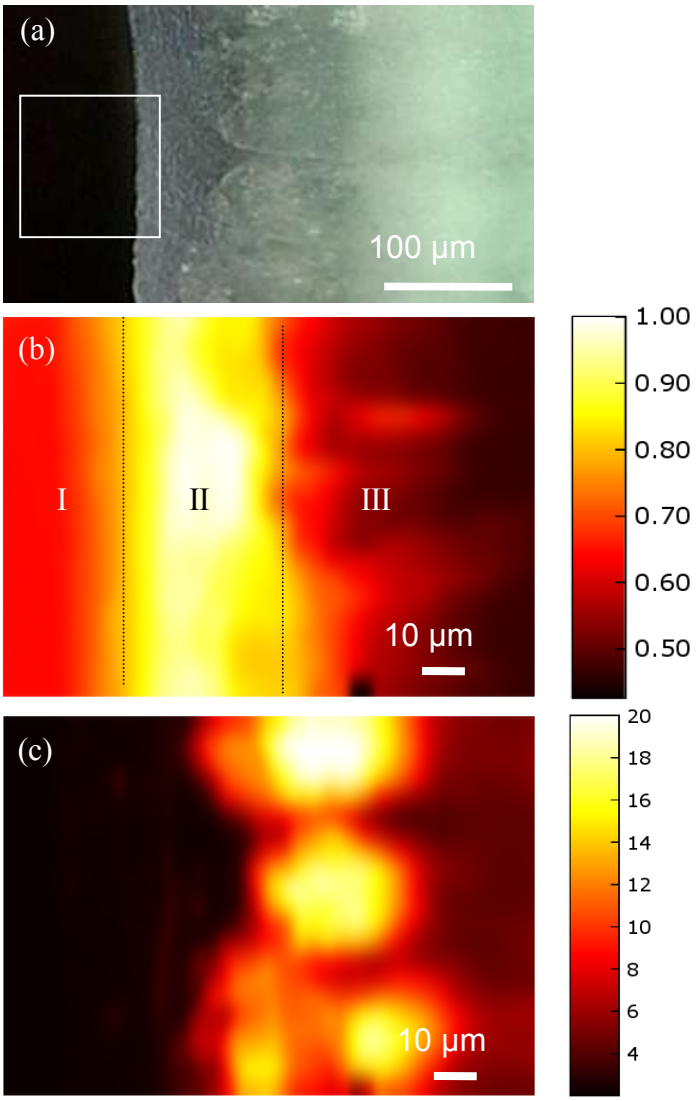

Figure 1. (a) Optical microscopy images of the inner surface of a 110 µm thick eggshell section showing the membrane on the left and mammillary spherulitic layer on the right; (b) X-ray transmission image of the region indicated by the box in (a) obtained using a 1 µm diameter beam with a scan step of 2 µm horizontally and 5 µm vertically; (c) integrated SAXS intensity (arbitrary units) of the same region [6].

The image of the integrated SAXS intensity (Fig. 1c) reveals strikingly different features in the form of three very intense microscopic focal discs stacked vertically. The upper and lower discs appear at the positions of the nucleating knobs observed in the optical image (Fig. 1a), close to the interface between the organic membrane (II) and mineral part (III). The difference of integrated intensity between the nucleating knob and the remaining mineralized layer was attributed to a decrease in the volume fraction of the organic content from the centre to the outside of the knob [7], as observed in other studies [8].

The second example is this of Bone, well known for it's sophisticated hierarchical architecture resulting in exceptional mechanical properties. At the fundamental level, bone is a composite composed of calcium phosphate nanoparticles at least partly embedded in collagen fibrils. At the next structural level, the fibrils are ordered into a variety of structures such as the osteons shown in Fig. 2a. These structures essentially result from the remodelling processes constantly renewing bone throughout the lifetime. They consist of cylindrical layers of ~ 3 µm in thickness which differ in the orientation of the collagen fibrils. However, the fine arrangement of the collagen macromolecules is still an open matter of debate. This is particularly important since it is well known that the mechanical properties of the osteons usually differ from the rest of the tissue.

L12

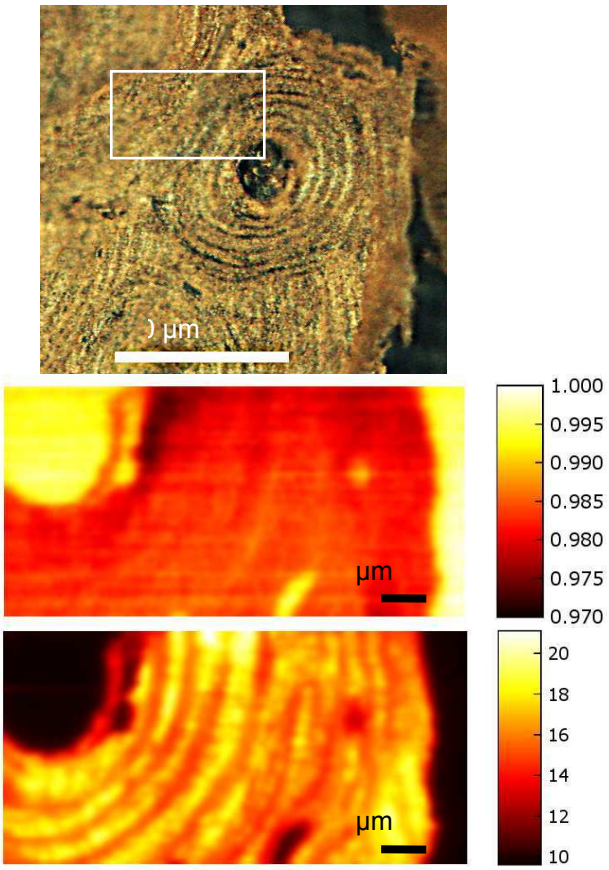

Figure 2. Optical microscopy images of a 5 µm thick human bone section taken in the dense cortical part of the femoral midshaft of a healthy human female; (b) X-ray transmission scan of the region indicated by the box in (a), obtained using a 1 µm diameter beam with 1 µm scan steps in both directions; (c) image of the SAXS intensity (arbitrary units) using the same scan parameters [6].

In the framework of a general study of the intra-lamellar structure, a thin sample section was analyzed under the same conditions as the eggshell. An area of 100×50 µm^2 (h×v) was covered in steps of 1 µm in both directions. The transmitted intensity shown in Fig. 2b appears to be uniform throughout the bone section which implies that the mineral density is constant. However, similar to the case of the eggshell, the image of the integrated SAXS intensity (Fig. 2c) clearly reveals features which could not be observed in the transmission image. In this case, alternating concentric rings appear, strongly reflecting the lamellar structure also seen in the light micrograph (Fig. 2a). The contrast in the SAXS image was attributed to changes in orientation of the mineral particles and thus the fibrils as demonstrated in other studies [9, 10].

These examples demonstrate the potential of this technique to image nanometer-sized heterogeneities in bulk samples. Although the main examples are biological materials, it will be shown that this method could be used for other domains of materials science.

References

[1] G. Jeronimidis, A.G. Atkins, "Mechanics of biological materials and structures – Nature's lessons for the engineer", *J. Mech. Eng. Sci.* **209** (1995) 221–235.

[2] P. Fratzl, "Biomimetic materials research: What can we really learn from nature's structural materials?", *J. R. Soc. Interface* **4** (2007) 637–642.

[3] S. Mann, S. Weiner, "Biomineralization: Structural questions at all length scales", *J. Struct. Biol.* **126** (1999) 179–181.

[4] H. Gao, B. Ji, I.L. Jager, E. Artz, P. Fratzl, "Materials become insensitive to flaws at nanoscale: Lessons from nature", *Proc. Nat. Acad. Sci. USA* **100** (2003) 5597–5600.

[5] C. Riekel, "New avenues in x-ray microbeam experiments", *Rep. Prog. Phys.* **63** (2000) 233-262.

[6] A. Gourrier, W. Wagermaier, M. Burghammer, D. Lammie, H.S. Gupta, P. Fratzl, C. Riekel, T.J. Wess, O. Paris "Scanning X-ray imaging with small-angle scattering contrast", *J. Appl. Crystallogr.* **40** (2007) 78-82.

[7] Y. Nys, M.T. Hincke, J.L. Arias, J.M. Garcia-Ruiz, S.E. Solomon, "Avian eggshell mineralization", *Poult. Avian Biol. Rev.* **10** (1999) 143-166.

[8] D. Lammie, M.M. Bain, S.E. Solomon, T.J. Wess, "Scanning microfocus small angle X-ray scattering study of the avian eggshell", *J. Bionic Eng.* **3** (2006) 11-18.

[9] W. Wagermaier, H.S. Gupta, A. Gourrier, O. Paris, P. Roschger, M. Burghammer, C. Riekel, P. Fratzl, "Scanning texture analysis of lamellar bone using microbeam synchrotron X-ray radiation", *J. Appl. Crystallogr.* **40** (2007) 115-120.

[10] W. Wagermaier, H.S. Gupta, A. Gourrier, M. Burghammer, P. Roschger, P. Fratzl, "Spiral twisting of fiber orientation inside bone lamellae", *Biointerfaces* **1** (2006) 1-5.