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Ultrafine heat-induced structural perturbations of bone mineral at the individual nanocrystal level

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ABSTRACT

The nanoscale characteristics of the mineral phase in bone tissue such as nanocrystal size, organization, structure and composition have been identified as potential markers of bone quality. However, such characterization remains challenging since it requires combining structural analysis and imaging modalities with nanoscale precision. In this paper, we report the first application of automated crystal orientation mapping using transmission electron microscopy (ACOM-TEM) to the structural analysis of bone mineral at the individual nanocrystal level. By controlling the nanocrystal growth of a cortical bovine bone model artificially heated up to 1000 °C, we highlight the potential of this technique. We thus show that the combination of sample mapping by scanning and the crystallographic information derived from the collected electron diffraction patterns provides a more rigorous analysis of the mineral nanostructure than standard TEM. In particular, we demonstrate that nanocrystal orientation maps yield valuable information for dimensional analysis. Furthermore, we show that ACOM-TEM has sufficient sensitivity to distinguish between phases with close crystal structures and we address unresolved questions regarding the existence of a hexagonal to monoclinic phase transition induced by heating. This first study therefore opens new perspectives in bone characterization at the nanoscale, a daunting challenge in the biomedical and archaeological fields, which could also prove particularly useful to study the mineral characteristics of tissue grown at the interface with biomaterials implants.

Statement of Significance

In this paper, we propose a new approach to assess the mineral properties of bone at the individual nanocrystal level, a major challenge for decades. We use a modified Transmission Electron Microscopy acquisition mode to perform an Automated Crystal Orientation Mapping (ACOM-TEM) by analyzing electron diffraction patterns. We tune the mineral nanocrystal size by heating a model bovine bone system and show that this method allows precisely assessing the mineral nanocrystal size, orientation and crystallographic phase. ACOM-TEM therefore has sufficient sensitivity to solve problems that couldn't be answered using X-ray diffraction. We thus revisit the fine mechanisms of bone nanocrystal growth upon heating, a process currently used for bone graft manufacturing, also of practical interest for forensic science and archaeology.

1. Introduction

Bone tissue is a biological nanocomposite material essentially composed of hydrated collagen fibrils of ~100 nm in diameter and up to several microns in length, reinforced by platelet-shaped nanocrystals of calcium phosphate apatite of $\sim 4 \times 25 \times 50\ nm^3$ in size [1]. These mineralized fibrils constitute the building blocks of bone tissue, and their specific arrangement is known to depend primarily on the dynamics of the formation and repair processes. Since these cellular processes can occur asynchronously in space and time, the mineralized fibrils adopt a complex hierarchical organization [1], which was shown to be a major determinant of the macroscopic biomechanical properties

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[2]. Extensive research programs are therefore currently focused on bone ultrastructure for biomedical diagnoses or tissue engineering applications.

However, structural studies at the most fundamental scales remain challenging due to the technical difficulties imposed by nanoscale measurements and by the tissue heterogeneity. Nevertheless, as a natural extension of bone mineral density (BMD) analysis, an important marker in current clinical studies, the following key characteristics of the mineral nanocrystals have been identified as potential markers of age and diseases: chemical composition, crystallinity vs disorder, crystal structure, size, shape and orientation [3,4]. Recent progress in the field showed that in order to obtain a deeper medical insight into the mechanisms of bone function, several such parameters need to be combined and correlated to properties at larger length scales [5]. Interestingly, from a totally different point of view, the archaeological community has drawn very similar conclusions concerning nanoscale studies for the identification, conservation and restoration of bone remains and artifacts [6].

From a materials science perspective, this is a well identified challenge in the analysis of heterogeneous nanostructured materials. Yet, technically, a major difficulty stems from the fact that most of the identified nanostructural bone markers require individual measurements on dedicated instruments which are generally difficult to combine in an integrative approach.

One 'gold standard' in nanoscale bone characterization is X-ray diffraction (XRD), which allows determining atomic-scale parameters averaged over the total volume illuminated by the X-ray beam. An important result from XRD studies conducted with laboratory instruments is that the bone mineral phase has, *on average*, a poorly crystalline apatite structure which, to a certain extent, is induced by a high fraction of carbonate substitutions [7]. Such studies enabled localization of a substantial number of elements other than calcium and phosphorus present in bone via ionic substitutions [8], which can lead to serious pathological conditions, e.g. skeletal fluorosis [9]. When an average description of bone properties is insufficient, synchrotron X-ray beams focused to a typical diameter of 0.1–10 μm [10–12] operated in scanning mode allow mapping the microstructural heterogeneities. However, this remains intrinsically an average measurement and the current instrumentation limits prevent any analysis at the single mineral nanocrystal level.

Transmission electron microscopy (TEM) is a second 'gold standard' in bone characterization at the nanoscale. In high resolution mode, it allows reaching sub-angström resolution [13] and therefore provides atomic details of the crystals. This increased resolution comes at the cost of the image field of view, which may not provide representative results due to the tissue heterogeneity. This limitation can partly be alleviated in scanning mode, which is more adapted to the collection of a large amount of data for statistical usage. In particular, for the process known as Automated Crystal Orientation Mapping (ACOM-TEM) [14], diffraction patterns are systematically acquired while the electron beam is scanning micron-sized areas, such that the structural parameters of hundreds of individual nanocrystals may be characterized and used to reconstruct orientation maps with nanometer spatial resolution.

To our best knowledge, the present study is the first reported use of the ACOM-TEM method to analyze mineral nanocrystals in bone tissue. To demonstrate the potential of this technique for bone studies, a test object is required which structure should be as close as possible to native bone, while offering a wide range of nanocrystal dimensions. Heated bone provides an ideal model for such purposes, ensuring a tight control over the nanocrystal size by adjusting the temperature. This system was extensively studied in archeological and forensic contexts. Upon heating to 100–150 °C, bone is progressively dehydrated [15] and collagen is considered to be fully degraded at ~400 °C [16,17]. Most X-ray studies concluded an absence of mineral crystal structure modifications before 400 °C, while a rapid crystal growth has been reported at ~750 °C [18–20]. In a recent study we provided evidence that the mineral nanocrystals increase in size and become more disorganized at temperatures as low as 100 °C [21]. In addition, many debates remain open concerning the nature of a postulated high-temperature phase transition, the co-existence of different crystallographic phases, as well as the presence of ionic defects above and below the critical temperature of T_{cr} ~750 °C [22]. The heated bovine cortical bone model therefore presents two main advantages to assess the potential of ACOM-TEM: 1) the possibility to fine-tune the mineral nanocrystal size upon heating and 2) the existence of a phase transition at high temperatures.

Using a set of bovine cortical bone samples in a control state and heated at eight temperatures ranging from 100 to 1000 °C, we show that ACOM-TEM provides enough sensitivity to probe fine crystalline modifications induced by heating, in particular, nanocrystal growth, subtle changes in stoichiometry and space group. These results provide new insight into the detailed effects of heating on bone and validate the use of ACOM-TEM for fundamental studies of the nanoscale organization of bone tissue in different contexts.

2. Materials and methods

2.1. Sample preparation

A bovine femur was obtained from the local slaughterhouse (ABAG, Fontanil-Cornillon, France). The medial cortical quadrant of a femoral section from the mid-diaphysis was extracted with a high precision circular diamond saw (Mecatome T210, PRESI) and fixed in ethanol 70% for 10 days (Supplementary information, Fig. S1). Nine $2 \times 2 \times 10$ mm^3 blocks were cut in the longitudinal direction and subsequently dehydrated (48 h in ethanol 70% and 100%) and slowly dried in a desiccator. One block was used as a control, while the others were heated to eight temperatures: 100, 200, 300, 400, 600, 700, 800 and 1000 °C for 10 min in vacuum (10^{-2} mbar) inside a quartz tube and cooled in air. The temperature precision of the thermocouple was ~2–3 °C and the heating rate was ~30–40 °C/min. The heating process resulted in color change, as shown in Fig. S2 of Supplementary information.

The samples were then embedded in poly-methyl methacrylate (PMMA) resin following the subsequent steps: impregnation, inclusion and solidification. For impregnation, a solution of methyl methacrylate (MMA) was purified by aluminum oxide and a solution of MMA was prepared with dibutyl phthalate in a 4:1 proportion (MMA1). The samples were kept at 4 °C in MMA1 for 5 days. For inclusion, the samples were stored in MMA1 solution with 1 wt% of benzoyl peroxide for 3 days and in MMA1 solution with 2 wt% of benzoyl peroxide for 3 days. The solidification took place in PTFE flat embedding molds covered by ACLAR film at 32 °C for 48 h. The resin-embedded blocks were then trimmed and cut with a diamond knife in a Leica UC6 ultramicrotome. The 50-nm-thick transverse sections (i.e., normal to the long axis of the femur) were deposited on 200 mesh Cu TEM grids coated with lacey carbon.

2.2. TEM data acquisition

The measurements were performed using a JEOL 2100F FEG-TEM (Schottky ZrO/W field emission gun) operating at an accelerating voltage of 200 kV and providing an electron beam focused to 2 nm in diameter at sample position. A camera was positioned in front of the TEM front window to collect diffraction patterns as a function of scanning position with a frame rate of 100 Hz. The