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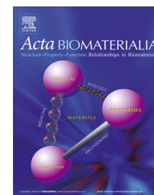

Imaging collagen packing dynamics during mineralization of engineered bone tissue

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ABSTRACT

The structure and organization of the Type I collagen microfibrils during mineral nanoparticle formation appear as the key factor for a deeper understanding of the biomineralization mechanism and for governing the bone tissue physical properties. In this work we investigated the dynamics of collagen packing during *ex-vivo* mineralization of ceramic porous hydroxyapatite implant scaffolds using synchrotron high resolution X-ray phase contrast micro-tomography (XPCμT) and synchrotron scanning micro X-ray diffraction (SμXRD). While XPCμT provides the direct 3D image of the collagen fibers network organization with micrometer spatial resolution, SμXRD allows to probe the structural statistical fluctuations of the collagen fibrils at nanoscale. In particular we imaged the lateral spacing and orientation of collagen fibrils during the anisotropic growth of mineral nanocrystals. Beyond throwing light on the bone regeneration multiscale process, this approach can provide important information in the characterization of tissue in health, aging and degeneration conditions.

Statement of Significance: BONE grafts are the most common transplants after the blood transfusions. This makes the bone-tissue regeneration research of pressing scientific and social impact.

Statement of Significance: Bone is a complex hierarchical structure, where the interplay of organic and inorganic mineral phases at different length scale (from micron to atomic scale) affect its functionality and health. Thus, the understanding of bone tissue regeneration requires to image its spatial-temporal evolution (i) with high spatial resolution and (ii) at different length scale.

Statement of Significance: We exploited high spatial resolution X-ray Phase Contrast micro Tomography and Scanning micro X-ray Diffraction in order to get new insight on the engineered tissue formation mechanisms. This approach could open novel routes for the early detection of different degenerative conditions of tissue.

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1. Introduction

Bone is a complex composite and nanostructured material formed via dynamic interactions between an organic matrix of biomacromolecules and an inorganic mineral phase [1,2]. The inorganic phase consists of carbonate apatite nanocrystals growing in the organic matrix composed by collagen (type I) proteins, with some minor noncollagenous proteins and minor amounts of lipids and osteogenic factors [3]. Collagen 3D arrangement constitutes a basic issue to be addressed since it plays a fundamental role in the bone tissue biomechanical properties. Collagen structure and organization has been investigated and visualized by several techniques such as magnetic resonance imaging (MRI) [4], ultrasound [5], electron [6] and optical imaging [7,8]. The obtained results

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indicate that the collagen molecule is a right-handed triple-helix, called *fibril*, about 300 nm long and 1.0–1.5 nm diameter. Collagen *fibers* are composed from about 100 up to more than 1000 tightly packed fibrils.

X-ray diffraction (XRD) is an effective tool for studying the fibrillar periodic assembly of collagen. Typically, X-ray scattering patterns from collagen present Bragg spots along the axial direction, indicative of a long-range ordered phase made of staggered parallel fibrils [9,10]. This results in a repeating axial D-spacing providing open sites for cross-links and mineral nucleation. Alongside the axial Bragg peaks, collagen XRD measurements show a diffuse equatorial scattering characteristic of a short-range order typical of fluid-like arrangement of the molecules [11–13]. Hulmes et al. interpreted XRD data as a molecular crystal with quasi-hexagonal molecular packing [14] while an arrangement in concentric layers was assumed by Parry et al. [15]. Doucet et al. [16] showed that the equatorial X-ray scattering patterns of collagen can easily be modeled using the paracrystal organization of fibers into parallel rods packed in a hexagonal or pseudo-hexagonal lateral network.

Unfortunately, conventional XRD as well as neutron diffraction [17] has a strong limitation since it provides insight only into the average concentration and structure of the collagen [12,13,18]. However collagen is, from a morphological point of view, a fluctuating and inhomogeneous tissue [19,20]: this makes quite difficult to model its structural features with the conventional experimental approaches, requiring high spatial resolution probes to map spatial changes in structural organization. In this framework the importance of the collagen axial D-spacing fluctuations has been recently recognized; indeed, significant alterations in bone collagen axial D-spacing distribution have been found in Osteogenesis Imperfecta [21] and long term estrogen depletion [22]. A quantitative method for space resolved axial D-spacing analysis at sub-micrometer scale, used Atomic Force Microscopy (AFM) and two-dimensional Fast Fourier Transform (2D FFT) analysis [23]. Anyway, being the AFM a surface approach, it does not allow to investigate the interplay of collagen spacing and minerals arrangement in the bulk composite material. Indeed, it is little known about the spatial distribution of both collagen and mineral crystals packing in the radial direction of the collagen fibrils. This is also due to the fact that conventional imaging techniques, such as electron microscopic tomography, can reach resolution of 4–6 nm that cannot resolve either the mineral nanocrystal size during the early stage of mineralization [24] either the collagen molecular lateral spacing (<2 nm) [9,25].

In this paper we overcome both the limitation of the 2D techniques and the limited resolution of the conventional tomography, by combining the 3D visualization of the collagen bundles provided by high resolution XPCμT with the high spatial resolution structural information obtained by SμXRD. This multiscale approach is needed due to the fact that collagen fibrils organize into bundles at micrometer scale via interfibrillar cross-links. At this scale, high resolution XPCμT is an appropriate tool to visualize collagen bundles since unlike conventional radiography and tomography it is able to image small density variations in weakly absorbing materials such as biological samples [26,27]. On the other hand, SμXRD technique allows to probe both the k-space and real space, visualizing structural features at atomic scale and nanoscale such as the lateral packing of collagen molecules and the size of mineral particles. The spatial mapping of the simultaneously collected Small Angle X-ray Scattering (SAXS) and Wide Angle X-ray Scattering (WAXS) signals, can readily monitor the mineralization process according to its temporal evolution. Although SμXRD has been already used for investigating ultrastructure in biological tissue, [28–30] in this work it is used to study the dynamics of the collagen fibrils during the HA nanocrystal nucleation and growth with a spatial statistical approach. More specifically, we measured the fluctuations of (i) the lateral spacing and preferred orientation of collagen fibrils (ii) the longitudinal and transverse size of mineral nanocrystals. Thus, the order degree of collagen arrangement and mineral growth during the bone tissue formation has been quantified by applying basics spatial statistics tools to the measured quantities on the large amount of collected data from different samples. This approach has been recently used to get key information on systems presenting structural fluctuations and heterogeneity on (sub)micrometric scales in different research fields ranging from material science to biomedicine [31–34].

2. Materials and methods

All experimental animal procedures were carried out in the IRCCS AOU San Martino –IST Animal Facility (Genoa, Italy), in the respect of the national current regulations regarding the protection of animals used for scientific purpose (D.lgsvo 27/01/1992, n. 116). Research protocols have been evaluated and approved by the IRCCS AOU San Martino –IST Ethical Committee for animal experimentation (CSEA) as Animal use project n. 336 communicated to The Italian Ministry of Health, having regard to the article 7 of the D.lgs 116/92.

Here we investigated collagen packing dynamics during nucleation and growth of bone mineral nanocrystals in ex vivo conditions: expanded bone marrow mesenchymal stem cells (BMSC) seeded onto porous ceramic scaffolds and subcutaneously implanted in the mouse [35]. After four weeks the scaffolds are removed from the host animals and the newly formed bone analyzed. To improve the statistical significance 5 samples extracted from different animals were studied.

2.1. Sample preparation

Marrow aspirates were obtained from the iliac crest of the experimental sheep as part of a protocol approved by the competent ethical authority. Bone marrow stromal cell (BMSC) cultures were counted with a nuclear stain, suspended in Coon's modified Ham's F12 medium supplemented with 10% FCS, 1 ng/ml of human recombinant FGF-2 and subsequently plated in 100-mm dishes at $0.5–1.0 \times 10^7$ cells per dish. Cultures were incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Before reaching confluence, cells were enzymatically detached and passaged. Experiments were performed using a pool of first passage BMSC derived from marrows of four animals. Osteogenic properties of the BMSC were evaluated by an "*in vivo*" assay in an immuno-deficient mice model. First passage cells expanded in standard medium were tripsynized, resuspended in a fibrinogen solution (Tissuecol; Baxter, Italia) to a final concentration of 62.5×10^6 cells/ml and loaded onto highly porous ceramic scaffolds (100% hydroxyapatite cubes, $3 \times 3 \times 3$ mm^3; FinCeramica, Faenza, Italy); an appropriate volume of 20 μl of Thrombin was added to ignite the enzymatic cleavage that originates a fibrin clot around and within the ceramic, entrapping the cells. Two samples were implanted subcutaneously in each mouse. After four weeks the samples dedicated to XμPCT were harvested, washed in Phosphate Buffered Saline (PBS) three times and fixed in paraformahaldehyde (4% in PBS) for 2–3 h at 4 °C. Additional washes in PBS removed the residual fixative. The samples for SμXRD were additionally dehydrated in ethanol at increasing concentration, embedded in methylmetacrilate and transversally cut using a diamond saw (Gillings- Hamco, Hamco Machines, Inc., Rochester, N.Y., U.S.A) in serial sections of about 100 μm thick. We choose 100 microns as a well suited thickness value, quite less than the general mineral bone attenuation length (about 350 μm at X-ray incident energy of 12.7 keV).

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