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X-ray micro-beam techniques and phase contrast tomography applied to biomaterials

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ABSTRACT

A deeper comprehension of the biomineralization (BM) process is at the basis of tissue engineering and regenerative medicine developments. Several in-vivo and in-vitro studies were dedicated to this purpose via the application of 2D and 3D diagnostic techniques. Here, we develop a new methodology, based on different complementary experimental techniques (X-ray phase contrast tomography, micro-X-ray diffraction and micro-X-ray fluorescence scanning technique) coupled to new analytical tools. A qualitative and quantitative structural investigation, from the atomic to the micrometric length scale, is obtained for engineered bone tissues. The high spatial resolution achieved by X-ray scanning techniques allows us to monitor the bone formation at the first-formed mineral deposit at the organic–mineral interface within a porous scaffold. This work aims at providing a full comprehension of the morphology and functionality of the biomineralization process, which is of key importance for developing new drugs for preventing and healing bone diseases and for the development of bio-inspired materials.

1. Introduction

Biomineralization (BM) is the widespread and fascinating process by which organisms form mineral materials, in organized crystals. Current research efforts in BM are aimed at the understanding of the underlying mechanisms that organisms use to control mineral formation. Only the full comprehension of the morphology and functionality of the biomineralized tissue (*i.e.*, shell, bone and teeth) will provide the opportunity to mimic nature for the development of bio-inspired materials and for the comprehension of degenerative diseases, such as osteoporosis and osteoarthritis. Among biomineralized tissues, bone has always received considerable attention, due to its great interest in the medical world and to its unique properties from a chemical and physical point of view. Indeed, besides the medical applications, e.g., dealing with tissue regeneration in case of fractures and various injuries [1], bone provides an important and intriguing model system to investigate the mechanisms involved in the formation of hierarchical self-assembled organic–inorganic composites [2–5] (from the molecular to the macroscopic level [6]). Indeed, bone is formed via dynamic interactions between supramolecular assemblies of biomacromolecules and forming mineral phases. The supramolecular assemblies consist of matrix proteins, mostly collagen (type I), with some minor non collagenous proteins and minor amounts of lipids and osteogenic factors (e.g., bone morphogenetic proteins). The mineral phase is composed of hydroxyapatite (HA) nanocrystals, a highly stable calcium phosphate. HA has been widely investigated in numerous areas, in order to achieve a better understanding of its formation mechanisms in natural biomineralization. This still constitutes a key point to be

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addressed for biomedical as well as industrial materials. To this end, monitoring what happens at the location in which organic–mineral interactions occurs becomes crucial.

Advanced experimental set-ups, like cryogenic Transmission Electron Microscopy (cryoTEM), electron tomography and advanced multi-Nuclear Magnetic Resonance (multi-NMR) spectroscopy have been recently used to perform in vitro and in vivo innovative measurements and to shade light to the BM mechanism in the bone [7,8]. Micro and nano X-ray diffraction are basic high-resolution tools, which allow for monitoring the evolution of the different organic and inorganic phases taking part in the process on a spatial scale from submicron to the order of 10 micron [9]. X-ray phase contrast tomography (XRPCT), on the other hand, provides the 3D spatial distribution of these different phases in newly formed bone and, in particular, the spatial distribution of soft tissue components, whose light densities cannot be properly appreciated by conventional tomography [10]. However, due to the complexity of the process, a complete and exhaustive explanation, still far from being reached, requires the synergy of different advanced experimental techniques. We report the possibility to study the BM process at different length scales using different and complementary X-ray techniques. We investigated the biomineralization process in an ectopic bone formation mouse model combining several high resolution techniques, namely: XRPCT, micro X-ray diffraction (μXRD) and micro X-ray fluorescence (μXRF). In this model, ex vivo expanded bone marrow mesenchymal stem cells are seeded onto a porous ceramic scaffold and implanted subcutaneously in the mouse [11]. After the established implantation time the scaffold is removed from the host animal and the newly formed bone is analyzed. XRPCT provided the 3D spatial distribution of the different phases: bone (B), scaffold (SC), and soft tissue (ST). On the other hand, the μXRD resolution in the reciprocal q-space allowed us to investigate, at the same time, the structural properties both at the nanometric and at the molecular atomic level. Moreover, scanning the sample with a micrometric beam allows for monitoring the early stage bone tissue development at different distances from the scaffold interface [12]. Finally, during the μXRD scanning, the calcium (Ca) distribution was also obtained by measuring the μXRF Ca signal.

2. Materials and methods

2.1. Sample preparation – Cell culture

Bone Marrow Stromal Cells (BMSC) were obtained from iliac crest marrow aspirates from healthy adult sheep (Italian Biellese strain; all donor animals had reached sexual maturity; animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies [13,14]). BMSC cultures were performed as previously described [14]. Briefly, nucleated cells 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 the marrow of four animals.

2.2. In vivo bone-forming efficiency

The osteogenic properties of the BMSC were evaluated by an "*in vivo*" assay in an immuno-deficient mice model [15]. First passage cells expanded in standard medium were trypsinized, 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; in turn, the latter originates a fibrin clot around and within the ceramic, entrapping the cells. Two samples were implanted subcutaneously in each mouse. After 8 weeks the samples were harvested, washed in Phosphate Buffered Saline (PBS) three times and fixed in paraformaldehyde (4% in PBS) for 2–3 h at 4 °C. Additional washes in PBS removed the residual fixative. One of the two samples was first decalcified and then embedded in paraffin, to evaluate bone formation by conventional histology. Hematoxylin–eosin staining was performed to reveal bone matrix deposition. The second sample was dehydrated in ethanol at increasing concentration, embedded in methylmethacrylate and, after the XRPCT measurements, transversally cut using a diamond saw (Gillings-Hamco, Hamco Machines, Inc., Rochester, N.Y., U.S.A) in serial sections (≈100 μm thick) for the μXRD measurements. After the μXRD measurements the slices were stained by Toluidine Blue to identify the regions occupied by newly formed bone tissue, soft connective tissue and bioceramic.

2.3. X-ray phase contrast tomography

The experiments was carried out at the TOMCAT beamline of the Swiss Light Source (SLS) in Villigen (Switzerland). We performed the experiment using an in-line set up. The sample does not need a specific preparation, but it was protected by a mylar foil during the measurements. The beam size was 2 mm at the sample, placed about 30 m downstream of the monochromator. The monochromatic incident X-ray energy was 17 KeV and a CCD camera with a pixel size of about 0.64 microns was used. Tomography data were acquired with 1601 projections covering a total angle range of 360°. A phase retrieval algorithm was applied to all projections of the tomographic measurements, using the ANKAphase code [16]. When applied to all tomographic projections, the retrieved phase maps can be fed to a standard filtered back-projection algorithm to obtain phase tomograms.

2.4. Micro X-ray diffraction and micro X-ray fluorescence

Scanning micro X ray diffraction and fluorescence experiments were simultaneously performed at the ID13 beamline of the European Synchrotron Radiation Facility (ESRF), Grenoble, France. The photon source, a 18 mm period in-vacuum undulator at ESRF works in the range 12–14 KeV with the storage ring operating at 6.03 GeV in the uniform mode with a current of 200 mA. The beamline uses a Si-111 channel cut crystal monochromator cooled with liquid nitrogen. A monochromatic X-ray beam of photon energy 14 keV ($\Delta E/E = 10^{-4}$) was used, which was focused by Kirkpatrick–Baez (KB) mirrors to a 1 μm spot size on the sample (full width at half maximum). A 16 bit two-dimensional Fast Readout Low Noise charged coupled device (FReLoN CCD) detector with 2048×2048 pixels of 51×51 mm^2 was used, binned to 512×512 pixels. The detector was placed 110 mm behind the sample and offset. The y–z scanning microdiffraction setup used a piezo-scanning stage with 0.1 micron repeatability. Diffraction patterns were recorded in transmission by a MAR CCD detector with a typical acquisition time of 10 s. 2D diffraction patterns could be radially integrated to provide 1D profiles of intensity vs. transfer moment q. μXRF signals were collected simultaneously by a Vortex silicon drift detector and a XIA electronic system providing an energy resolution of 10 eV per channel. The experimental set up is reported in Fig. 1.