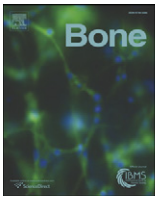
Bone 50 (2012) 301–310

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone



Original Full Length Article

Ultrastructure of regenerated bone mineral surrounding hydroxyapatite–alginate composite and sintered hydroxyapatite

Andre L. Rossi [a], Isabela C. Barreto [b], William Q. Maciel [a], Fabiana P. Rosa [b], Maria H. Rocha-Leão [c], Jacques Werckmann [a], Alexandre M. Rossi [d], Radovan Borojevic [a], Marcos Farina [a],*

[a] Institute of Biomedical Sciences, Federal University of Rio de Janeiro (UFRJ), Brazil
[b] Institute of Health Sciences, Federal University of Bahia, Brazil
[c] School of Chemistry, UFRJ, Brazil
[d] Brazilian Center for Physics Research, CBPF, Brazil

ARTICLE INFO

Article history:
Received 24 March 2011
Revised 21 September 2011
Accepted 21 October 2011
Available online 28 October 2011

Edited by: David Fyhrie

Keywords:
Bone implant
Hydroxyapatite–alginate composite
Bone ultrastructure
Biomaterials
Biomineralization
HRTEM

ABSTRACT

We report the ultrastructure of regenerated bone surrounding two types of biomaterials: hydroxyapatite–alginate composite and sintered hydroxyapatite. Critical defects in the calvaria of Wistar rats were filled with micrometer-sized spherical biomaterials and analyzed after 90 and 120 days of implantation by high-resolution transmission electron microscopy and Fourier transform infrared attenuated total reflectance microscopy, respectively. Infrared spectroscopy showed that hydroxyapatite of both biomaterials became more disordered after implantation in the rat calvaria, indicating that the biological environment induced modifications in biomaterials structure. We observed that the regenerated bone surrounding both biomaterials had a lamellar structure with type I collagen fibers alternating in adjacent lamella with angles of approximately 90°. In each lamella, plate-like apatite crystals were aligned in the *c*-axis direction, although a rotation around the *c*-axis could be present. Bone plate-like crystal dimensions were similar in regenerated bone around biomaterials and pre-existing bone in the rat calvaria. No epitaxial growth was observed around any of the biomaterials. A distinct mineralized layer was observed between new bone and hydroxyapatite–alginate biomaterial. This region presented a particular ultrastructure with crystallites smaller than those of the bulk of the biomaterial, and was possibly formed during the synthesis of alginate-containing composite or in the biological environment after implantation. Round nanoparticles were observed in regions of newly formed bone. The findings of this work contribute to a better understanding of the role of hydroxyapatite based biomaterials in bone regeneration processes at the nanoscale.

Introduction

The clinical success of an implant is dependent on the juxtaposition and vitality of the new bone tissue regenerated around the biomaterials as well as on the integrity of the bone–implant interface, which acts as an anchoring surface for the collagen fibers that are formed later [1,2]. Several biomaterials and biocomposites are being used in the field of bone engineering [3–8]. However the behavior of these materials after implantation and the structural characteristics of the newly formed tissue surrounding the materials at the nanoscale, are not completely known. The way new bone is structured and adhered to the surface of the biomaterial and the potential distinct mineralized phases present, should be studied before clinical applications, aiming to the materials optimization. Hydroxyapatite has been used alone, or in association with cells, growth factors and polymers in a wide range of medical applications because of its biocompatibility, bioactivity and osteoconduction properties [2,3,9].

The association of hydroxyapatite with alginate is a promising composite, widely applied in biomedical engineering to form porous mineralized scaffolds after calcination [10–12]. Alginate is a natural polysaccharide extracted from seaweed and bacteria. The addition of divalent cations, such as calcium, to alginate in aqueous solution may induce cross links between molecules and the formation of hydrogels, a tridimensional network that permanently stabilizes the polymers.

In a previous work [13], we showed that sub-millimeter spheres of non-calcined hydroxyapatite–alginate (HA-Alg) composite used as grafts in critical defects of rat calvaria promoted bone formation in different length scales. The apparent bioactive behavior of the biomaterial could be related to the alginate phase of the composite that forms highly anionic polymers (rich in carboxylates) with a potential to adsorb specific molecules from the extra-cellular matrix and thus induce osteoblast differentiation locally. However, the evolution of

---

* Corresponding author at: Laboratório de Biomineralização, ICB, CCS, UFRJ, CEP 21941-902, Rio de Janeiro, RJ, Brazil.
E-mail address: mfarina@anato.ufrj.br (M. Farina).

8756-3282/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2011.10.022

this new composite after several weeks of implantation, the consequences to the bone–biomaterial interface and the ultrastructure of the regenerated bone surrounding HA-Alg had not been evaluated at the nanometer scale [13].

Similar to other mineralized tissues, bone is a hierarchically structured composite. It is formed by apatite crystals and organic components, of which type I collagen represents 90% [14,15]. Depending on the bone type and the species, different arrangements of collagen fibrils and apatite crystals have been reported [16].

Each type of fibril arrangement has mechanical specificities and advantages, which are related to the bone function [16]. Compared to parallel-fibered and radial-fibered arrangements, the plywood model in the osteonal system and lamellar bone is mechanically optimized to support stress from any direction applied [16–18]. Different types of plywood-like structures have been described for bone: orthogonal plywood [19], twisted plywood [19] and rotated plywood [20].

Woven bone is formed by loosely oriented fibers arranged in bundles with little evidence of intra-fibrillar mineralization [21]. Its rate of mineralization is faster than that of lamellar bone. Woven bone is the first bone formed during initial bone formation in fracture and pathologic circumstances [21].

The first mineralized layer formed at the interface bone–biomaterial is a cement layer-like structure, which is analogous to the cement layer in the contour of a new haversian system formed by the remodeling process of bone [1,22]. This layer is free from collagen and presents an organic matrix rich in bone sialoprotein and osteopontin that provides nucleation and modulation sites for calcium phosphate mineralization [1].

Bone mineralization is a cell-regulated process that maintains the morphology and strength of the tissue [23] and involves an organic matrix, specific proteins and a system for transporting ions to the mineralizing site [24]. Different mechanisms for the nucleation of bone crystals have been proposed: (1) osteoblast cells may secrete membrane-bound matrix vesicles (50–200 nm in diameter) into the osteoid tissue by budding. Hydroxyapatite nanocrystals grow inside these vesicles and eventually disrupt the vesicles forming mineralized nodules in the extracellular matrix [23]; (2) organic components from the extracellular matrix may be directly related to nucleation of hydroxyapatite crystals and nodules formation, independently from the matrix vesicles. These structures are known as crystal sheaths or crystal ghosts [25]; (3) mineralization of bone apatite crystals is mediated first by extracellular matrix vesicles and later by type I collagen fibrils, both inside type I collagen fibrils or onto the fibrils independently from the matrix vesicles mineralization process [26,27]. By analyzing the mineralizing leg tendon from the domestic turkey by electron tomography, Landis et al. [28] found that the mineral phase inside gaps of the fibrils was present as small irregularly shaped platelets with the crystallographic *c*-axes pointing parallel to the collagen long axes.

Understanding the mechanism by which bone regenerates around new composite biomaterials is important for developing and optimizing new biomaterials for bone engineering as well as for understanding the influence of the different biomaterials in bone formation. The architecture of bone, including the morphology, dimensions and distribution of the nanocrystals in the cement layer and collagen fibrils should be investigated in detail to assess their crystallographic characteristics and their role in bone strength and mechanical properties around an implant. Thus, the motivation for this work was to find a possible correlation between nanostructure at the interface biomaterial–newly formed bone, and characteristics of regenerated bone at the meso and macro-scales.

In this work we investigated the mineralized material surrounding HA-Alg composite and sintered hydroxyapatite (HA-Sint) used to regenerate critical bone defects in rat calvaria by high-resolution electron microscopy (HRTEM)/electron diffraction and Fourier transform infrared attenuated total reflectance microscopy (FTIR). Our results revealed differences mainly in the new mineralized regions in close proximity to the implanted biomaterials.

Materials and methods

Biomaterials

Synthetic hydroxyapatite powder was mixed with sodium alginate (Keltone) in a 15:1 proportion. The mixture was dropped from a syringe into a solution of calcium chloride (0.15 mol/L $CaCl_2$) at room temperature to produce a composite of small sub-millimeter-sized spheres containing polymers of calcium alginate mixed with the nanoparticles [13,29]. The spheres were removed from solution and washed in distilled water. Half of the sample was air dried and stored at room temperature (HA-Alg). The dried spheres maintained the same HA/Alginate content and similar dimension than as-prepared spheres. The other half of the sample was calcined in a muffle under oxygen flux at 1100 °C (HA-Sint). The thermal treatment was conducted to: (i) eliminate alginate from the spheres ($T>240$ °C) and (ii) induce the sintering of hydroxyapatite particles ($T>800$ °C). The sintering process increases the mean particle size, drastically reduces the inter-particle spaces and produces sample densification. In our case, the thermal treatment at 1100 °C produced an efficient densification of HA particles: the mean particle size increased from its non annealed value while the diameters of the spheres decreased.

Spheres containing alginate and sintered hydroxyapatite were sieved to produce samples with diameters ranging from 400 to 600 μm. Before implantation in rats, biomaterials were analyzed by TEM (Transmission Electron Microscopy) and SAED (Select Area Electron Diffraction). The spheres were embedded in epoxy resin, ultrathin sectioned (ca. 70 nm thick) using an ultramicrotome (PT-XL PowerTome, RMC) and analyzed in a JEOL 1200-EX electron microscope. SAED from biomaterials were obtained with constant camera length and an accelerating voltage of 100 kV. TEM imaging and diffraction were previously calibrated with a gold standard (Ted Pella, Inc).

Biomaterial implantation

For microscopy studies we used 12 male rats (*Rattus norvegicus*), 6 rats for each biomaterial, besides the control group. The rats were between 3 and 4 months old, with body weights ranging from 350 to 420 g. Critical circular defects of circa 8 mm in diameter and 1.5 mm deep were made in the calvaria between anterior and posterior cranial sutures using a trephine milling cutter under constant irrigation and were filled with HA-Alg or HA-Sint biomaterials. Anesthesia was given by intramuscular injection of ketamine chloride (0.1 mL/100 g of body mass) and sedation plus analgesia was given by intramuscular injection in a single dose of xylazine chloride (0.04 mL/100 g body mass). After 90 days of implantation, rats were killed with a lethal dose of ketamine chloride and the samples containing the defect were removed from the calvaria. This work was approved by the Ethical Committee on the Use and Care of Animals of the State University of Feira de Santana (Bahia-Brazil). For Fourier transform infrared spectroscopy (FTIR) additional 2 rats were used and analyzed after 120 days.

Sample preparation and light microscopy observations

After 90 days of implantation, samples containing the circular defect in calvaria with a small portion of original bone in the periphery were obtained surgically and sectioned by sagittal planes. Samples were fixed for 48 h in a solution containing glutaraldehyde (2.5%) and paraformaldehyde (4%) in cacodilate buffer (0.1 M, pH 7.3). Post-fixation was performed with osmium (1%) in cacodylate buffer