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In vivo osteointegration of three-dimensional crosslinked gelatin-coated hydroxyapatite foams

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ABSTRACT

The main requirement of bone regenerative scaffolds is to enhance the chemical reactions leading to the formation of new bone while providing a proper surface for tissue in-growth as well as a suitable degradation rate. Calcium phosphate ceramics are conformed by different shaping methods. One requirement is to design implants and scaffolds with suitable shapes and sizes, but also with interconnected porosity to ensure bone oxygenation and angiogenesis. In this work we present the in vivo performance of hierarchically arranged glutaraldehyde crosslinked, gelatin-coated nanocrystalline hydroxyapatite (HABP) scaffolds (1–400 μm), with high potential as bone regenerators and excellent osteointegration performance, as well as an appropriate bioresorption rate. 6 × 10 mm bone defects were made in the lateral aspect of both distal femoral epiphysis of 15 mature (9 months old) male New Zealand rabbits. The bone defect in the left femur was then filled by using HABP foam cylinders, allowing the surgeon to carve the appropriate shape for a particular bone defect with high stability intra-operatively. The foam becomes swollen with body fluid and fills the cavity, ensuring good fixation without the need for a cement. Histological and radiographical studies after 4 months implantation showed healing of all treated bone defects, with bone integration of the HABP foam cylinders and bone conduction over the surface. This in vivo behaviour offers promising results as a scaffold for clinical applications, mainly in orthopaedics and dentistry.

1. Introduction

Calcium phosphates are widely used as biocompatible bone fillers and regenerators in tissue engineering, as they are very similar in hardness to natural bone tissue. These types of ceramics are conformed by different synthesis and shaping methods. The main requirement of these scaffolds is to be precursors of newly formed bone as well as to ensure bone oxygenation and angiogenesis [1]. For this purpose the designed materials must have interconnected porosity which must also include a number of macropores [2]. Reported pore sizes supporting the osteointegration of implants have been in the range 1–500 μm for a variety of biomaterials, such as hydroxyapatite (HA), β-calcium phosphate and biphasic calcium phosphate [3,4]. It is also necessary to find a compromise between a suitable degradation rate and a robust scaffold inducing tissue regeneration. These materials have attracted wide interest because of their excellent biocompatibility and bioactivity, having a positive influence on the biological responses of cells.

Some of the modern approaches to improving the performance of metallic, polymeric and ceramic scaffolds have been to attach growth factors to the scaffold surface [5–7] or to seed them with osteoprogenitor cells to facilitate osteoblast colonization and induce cell penetration over the three-dimensional volume [8,9]. Moreover, studies on the influence of the degree of porosity on scaffold resorption have shown it to be a key parameter in the balance of all parameters involved in bone reconstruction [10]. Nevertheless, it is expected that the simpler the scaffolds design, the simpler would be the manipulation during the surgical procedure. Because of this, the synthesis of hierarchical porous scaffolds has been under examination for several decades and remains so today, with the perfect combination of all requirements still under development.

Three-dimensional (3-D) hydroxyapatite (HA) foams with a high degree of porosity were prepared by sol–gel routes, and in order to confer flexibility and handleability the foams were coated with gelatin–glutaraldehyde biopolymers. These foams have previously been proposed by our group as new potential devices for the treatment of heavy metal intoxication by ingestion and as water

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purifiers [11,12]. However, both the composition and 3-D interconnected architectural design of these HA foam-like systems suggest that they would allow cellular internalization and colonization over the entire surface. Thus an exhaustive study of the in vitro response of osteoblast-like cells and the in vitro degradation process was presented in a previous work, exhibiting excellent in vitro performance and a suitable degradation rate with non-cytotoxic degradation products [13].

The design of these scaffolds allows rapid bone in-growth together with fast ceramic resorption as well as easy manipulation during surgery.

Herein, for the first time, the potential of these HABP foams as bone regenerators in vivo has been studied in 15 mature New Zealand rabbits. For this purpose radiological and histological studies have been performed to asses the effects of the scaffolds on osteointegration.

2. Experimental

2.1. Scaffold fabrication and characterization

3-D HA foams have been synthesized and conformed in a one-step process following a sol–gel technique [11], including the non-ionic surfactant Pluronic F127 ($EO_{106}PO_{70}EO_{106}$) as a macropore inducer using an accelerated evaporation-induced self-assembly (EISA) method [12]. HA was obtained from the reaction of calcium nitrate tetrahydrate and triethylphosphite (TIP) (Aldrich, Steinheim, Germany) at a molar ratio of F127:TIP of 11. The resulting foams were coated by immersion in a solution of a biocompatible polymer in use in US Food and Drug Administration (FDA) approved products (1.2% w/v type A gelatine (porcine skin) crosslinked with 0.05% w/v glutaraldehyde) as reported elsewhere [11]. Scanning electron microscopy (SEM) in a JEOL 6400 microscope (Tokyo, Japan) was used to characterize the macroporous 3-D architecture of the biopolymer-coated HA foams (HABP). Hg porosimetry measurements were carried out in an AutoPore III porosimeter (Micromeritics Instrument Corp., Norcross, GA) and X-ray diffraction (XRD) in a Philips X'Pert diffractometer using Cu K_α radiation. Further details of the foam characterization have been reported previously [11,14].

2.2. Surgical procedures

15 male mature New Zealand rabbits, 9 months old and weighing on average 3.751 g (3.231–4.450 g) were used. The animals were placed in individual cages, under standard conditions (room temperature 20 ± 0.5 °C, relative humidity 55 ± 5% and illumination with a 12 h/12 h light/dark photoperiod), fed with full rabbit special fodder (Nantas®), given water ad libitum, and were without restriction of movement. Surgery was performed under aseptic conditions and general anaesthesia induced by injection of ketamine (75 mg kg^{-1}), supported by an inhalation mask of O_2 and isoflurane (2.5 and 0.8–1.5 l min^{-1}, respectively). Analgesia was maintained by subcutaneous injection of buprenorfine (0.001–0.05 mg kg^{-1}), and antibiotic prophylaxis was by means of two injections of cefazoline (50 mg kg^{-1}). Using a motorized drill a bone defect 6 mm in diameter and 10 mm in depth was made in the lateral aspect of both distal femoral epiphyses in all animals, using continuous irrigation with physiological saline to prevent bone necrosis. The bone defect in the left femur was then filled using HABP foam cylinders previously carved into the required shape with a scalpel and sterilized with ethylene oxide. The bone defects in the right femora served as controls. The designed bone defects corresponded to critical bone defects according to the standard requirements [15–19]. All the animals were killed pharmacologically under general anaesthesia after 4 months. The 15 rabbits formed one group and were numbered 1–15.

2.3. Radiographical and histological evaluation

During follow-up standardized lateral digital radiographs were taken monthly (high ionization Sedecal tube, 400 mA, 500 mAs, 150 kV, indirect digital radiography, Fujifilm digital reader, FCR Prima).

After death the femora were extracted for histological study. Three 3 mm thick longitudinal sections from both femurs were obtained from each animal and placed in a buffered paraformaldehyde (10%) solution for fixation by immersion for 15 days. After fixation the samples were washed and decalcified in 5% nitric acid solution for 10 days.

For the histological study the samples were dehydrated through a graded ethanol series (70, 96 and 100%) and cleared with xylene, before being embedded in paraffin using a Leica TP 1050 tissue processor. The paraffin blocks were cut into 4 μm sections with a Shandon Finesse 325 microtome, deparaffinized and rehydrated. The sections were stained with hematoxylin and eosin (H&E) and Masson–Goldner Trichrome for histological evaluation.

Histological sections were examined with a Zeiss AxiokoP 40 microscope and microphotographs were obtained with a Zeiss AxioCam MRc5 camera.

All procedures were carried out under Project Licence PI45/10 approved by the in-house Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

3. Results

3.1. HABP foam characterization

HABP foams have a controlled distribution of interconnected macroporosity in the range 1–400 μm, determined by Hg intrusion porosimetry and observed by SEM in Fig. 1a and c. Low temperature sol–gel synthesis allows crystal size control and results in a nanocrystalline structure, as confirmed by the XRD pattern corresponding to pure nanocrystalline HA (ICDD PDF 9-432). The average crystallite size was calculated based on the 100 and 001 reflections by Rietveld refinement [20] giving a value of 20 nm. As shown in Fig. 1c, the Hg intrusion porosity technique performed before and after biopolymer coating (an SEM image of the coated HABP foam is shown in the inset, with a pore volume value of 70%) shows only a slight decrease in the total pore volume distribution in the range 100–300 μm [11]. The amount of crosslinked gelatin on the coated HA foams was determined by thermogravimetric analyses of three replicates, showing a biopolymer content of 20 ± 2% (see Supplementary Fig. S1). As has been reported previously, crosslinked gelatin-coated foams have a swelling ratio of 400*W*% when immersed in aqueous solution due to the hydrophilic nature of gelatine crosslinked with glutaraldehyde [11] (see Fig. 1d and Supplementary Fig. S2). Swelling was calculated as *W* (swelling) (%) = $100 \times (W_t - W_d)/W_d$, where W_d is the weight of dried foam and W_t is the weight of hydrated foams at time t (0–24 h).

3.2. Intra-operative findings

The carved HABP foam cylinders were easily managed during surgery and remained stable in the bone defect. After surgical implantation into the bone defect the carved HABP foam cylinders