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ScienceDirect

Ceramics International 40 (2014) 771–777

CERAMICS INTERNATIONAL

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Characterization of bovine-derived porous hydroxyapatite scaffold and its potential to support osteogenic differentiation of human bone marrow derived mesenchymal stem cells

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Received 5 April 2013; received in revised form 29 May 2013; accepted 19 June 2013
Available online 9 July 2013

Abstract

Porous three-dimensional hydroxyapatite (HA) scaffolds were prepared using bovine cortical bone derived HA (BDHA). Analyses of the morphology, chemical composition, and phase purity of the scaffold were performed using scanning electron microscopy (SEM), micro-computer tomography (micro-CT), Fourier transform infrared spectroscopy (FTIR), energy dispersive X-ray spectroscopy (EDX), and X-ray diffraction (XRD). SEM images revealed the rough and porous surface of the scaffold, while micro-CT showed the average porous volume of $76.7 \pm 0.6\%$ and pore size of 0.04–0.25 mm. Single phase corresponding to standard HA was observed using XRD, and FTIR confirmed the presence of functional groups similar to HA. The EDX analysis revealed a Ca/P ratio of 1.61, which was comparable with HA stoichiometry. Compressive strength of the BDHA scaffold was found to be 1.3 ± 0.09 MPa. After 14 days of human bone marrow stromal cells (hBMSCs) seeding, SEM and confocal analysis revealed cell attachment to the surface and infiltration into the pores. Alamar blue and alkaline phosphatase assays showed significantly increased cell proliferation and differentiation in the BDHA scaffold, when compared with that in the monolayer ($p < 0.01$). In addition, quantitative real-time polymerase chain reaction (qPCR) data confirmed the up regulation of genes involved in osteogenic differentiation of mesenchymal stem cells. Our findings indicate that BDHA scaffold provides a favorable physiological environment for enhanced cell attachment, proliferation, and osteogenic differentiation of hBMSCs.
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Keywords: A. Sintering; E. Porosity; Hydroxyapatite; Mesenchymal stem cell

1. Introduction

The use of three-dimensional (3D) scaffolds has been commonly accepted as an essential constituent in bone tissue engineering [1]. As such, various types of scaffolds – natural, synthetic, or a combination of both – have been developed and numerous potential materials for preparing these scaffolds have been introduced. These scaffolds create and maintain space that facilitates progenitor cell migration, proliferation, and differentiation. However, a dispute exists regarding the structural and mechanical properties of scaffolds [2], because the scaffold is expected to remain osteoconductive and subsequently degrade as native tissue forms at the defect region [3]. Current advanced technology in polymer engineering allows fabricating or tailoring synthetic polymer scaffolds such as polycarbonates-, polyphosphazenes-, polyanhydrides-, and polyethylene glycol (PEG)-based hydrogels to satisfy the needs of bone regeneration [4]. Synthetic polymers such as PLA and PGA have been reported to produce toxic solutions and small particles due to acidic degradation [5]. These small particles trigger an inflammatory response as they are phagocytized by macrophages [6]. In clinical studies where PGA was used as fracture fixation, foreign-body responses or osteolytic reactions have been reported [7,8]. Therefore such issues affect biocompatibility of the implanted material.

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http://dx.doi.org/10.1016/j.ceramint.2013.06.067

Furthermore, advanced computer-aided design and computer-aided manufacturing (CAD–CAM) technologies have allowed developing 3D design (3-dimension) model of these scaffolds with fine-tuned micro and macroarchitectures [9,10]. Nevertheless, there are issues involved in manufacturing of these scaffolds. First, customized rapid prototyping machine to prepare these bone grafts is very expensive and only available in accredited material engineering centers. Second, the skills and techniques involved in handling this machine are highly advanced and costly. As a result, the cost of the end product is exorbitant, making it available only in affluent medical centers [11]. Therefore, an easily available and cost-effective scaffold material is imperative. Hydroxyapatite (HA), a predominant mineral component of bone, has been widely used as a potential candidate for bone tissue engineering [12]. It is regarded as a biocompatible and biodegradable material because it tends to integrate well into host tissue and degrades over the time without eliciting an immune response [13]. Recently, its application as a scaffold in cell-based therapy has been examined. For the development of a successful scaffold, 3D interconnected porous structure has been reported to be necessary to allow cell attachment, proliferation, and differentiation. Thus, in the present study, we investigated the proliferation and differentiation potential of human bone marrow derived mesenchymal stem cells (hBMSCs) in bovine-derived porous HA (BDHA) scaffold.

2. Experimental

2.1. Fabrication

HA was extracted from the femur of adult bovine bone as described previously [14]. The extracted HA was mixed with 30 wt % commercial sugar (sieved with 300-μm sieve plates to obtain particles of size below 300 μm). The powder mixed with sugar was uniaxially compacted at 156 MPa into green bodies using 10-mm cylindrical dies. Pressureless sintering at atmospheric pressure and ambient humidity was performed on pre-prepared green bodies using a furnace box (Elite Thermal System Ltd., BSF12/6-2408CP) in air atmosphere at 900 °C and ramp rate of 5 °C/min, and dwell time of 2 h. During the sintering process, the green bodies were placed in alumina crucibles (Coors high alumina, Sigma) without covers. The cylindrical BDHA scaffold was used for physicochemical characterization ($n=6$), pore size and porosity characterization ($n=6$), biomechanical ($n=6$), cell attachment ($n=3$), cell culture ($n=8$) and gene expression analysis ($n=8$).

2.2. Characterization

The XRD patterns of BDHA powder were recorded on a D8 Advance X-Ray diffractometer (Bruker-AXS, USA) using Ni-filtered monochromatized CuK_α radiation at 40 kV and 40 mA at 25 °C. The diffractogram of HA was found to be correlated with the Joint Committee on Powder Diffraction Standards (JCPDS) diffractogram values of HA (Ca_{10} $(PO_4)_6(OH)_2$) obtained from PDF-4 database (COMBICAT, University of Malaya). High beam of charged particles of approximately 10–30 kV was focused on the specimens using EDX (INCA Energy 200, Oxford Instruments). The number and energy of the X-rays emitted from these specimens were measured by an energy-dispersive spectrometer using Si (Li) crystal detector, and the EDX spectrum was plotted using Micro-analysis Suite software (Version 4.05-Oxford Instruments). For FTIR, BDHA scaffolds were crushed and pressed to obtain thin circular wafer, and the transmission spectra were recorded at 4000–400/cm range. A micro-CT system (SkyScan 1076, Belgium) was used to quantify the 3D microstructural properties of the BDHA scaffold. Isotropic slice data were obtained by the system and reconstructed into two-dimensional images, sliced, compiled, and analyzed to produce 3D images for quantitative architectural parameters [15]. Porosity, pore sizes, and pore distribution were measured from the constructed 3D model.

2.3. Mechanical property

Unconfined isostatic compression test was carried out at a loading speed of 10 mm/min between parallel steel plates using uniaxial compression machine (Instron model 3365, USA). Cylindrical BDHA specimens were prepared with an average dimension of 8.38 ± 0.01 mm (diameter) and 11.86 ± 0.02 mm (height). Stress–strain curve following compression of the BDHA scaffold was generated using Instron software version Blue Hills 2.

2.4. Mesenchymal stem cells culture and assay

hBMSCs were harvested and cultured as described previously [16]. 1×10^6 cells/ml were seeded onto monolayer and porous BDHA scaffold and cultivated with osteogenic medium (Gibco, Invitrogen, USA). Viability assay was carried out based on percentage of alamar blue (AB) reduction on day 0, 3, 6, 9, 12, 15, and 21. Cell attachment was analyzed on day 14 by SEM and confocal microscopy using Hoechst 33342 nuclear stain. Osteogenic differentiation of hBMSCs was measured by alkaline phosphatase (ALP) activity and osteocalcin (OC) secretion on different time points (day 0, 3, 6, 9, 12, 15, and 21).

2.5. Gene expression

The total RNA was isolated from BDHA and monolayer cultures on days 14 and 21 after culturing in osteogenic medium. The RNA isolated from untreated MSCs was considered as a baseline for all the groups. The samples were washed with 1× PBS and incubated for 4 h in Trizol reagent, and then vortexed vigorously. The supernatants of all the sample groups were collected and the RNA from the collected supernatants was isolated using an RNeasy Mini Kit, according to manufacturer's instructions. Subsequently, 1 μg of RNA was used to generate cDNA using the Superscript III First Strand Synthesis Kit, according to manufacturer's instructions. Primers for alkaline phosphatase (ALP) and osteocalcin (OC) were designed using NCBI database (USA) prior to q-PCR (Table 1). After an initial denaturation step at 95 °C for 3 min, the cDNA products were amplified with 40 PCR cycles, consisting of a denaturation step at 95 °C for 30 s, annealing temperature ranging from 50 to 60 °C, and an extension step at 72 °C for 5 min. The relative quantification values were analyzed using the Bio-Rad CFX manager 2.0. The level of expression of each target gene, normalized

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