Available online at www.sciencedirect.com
ScienceDirect
Acta Biomaterialia 5 (2009) 1725–1731
Acta BIOMATERIALIA
www.elsevier.com/locate/actabiomat

Preparation of porous bioactive ceramic microspheres and in vitro osteoblastic culturing for tissue engineering application

S.-J. Hong [a], H.-S. Yu [a], H.-W. Kim [a,b,*]

[a] Department of Biomaterials Science, School of Dentistry, Dankook University, Cheonan, 330-714, South Korea
[b] Institute of Tissue Regeneration Engineering (ITREN), Dankook University, Cheonan, 330-714, South Korea

Received 4 June 2008; received in revised form 11 November 2008; accepted 5 December 2008
Available online 25 December 2008

Abstract

Microparticulates are useful for directly filling defective tissues as well as for delivering cells and bioactive molecules in regenerative medicine. This paper reports on the production of bioactive ceramic microspheres with an interconnected macropore structure. The sol–gel derived calcium silicate powder was homogenized with an oligomeric Camphene melt, which was used as a novel porogen, and spherical-shaped microparticulates were obtained by an oil-in-water emulsion method. A porous structure was generated through the sublimation of Camphene within the calcium silicate–Camphene solidified blend under ambient conditions. The microspheres retained the crystalline phase of apatite and wollastonite during heat treatment and induced calcium phosphate precipitation under a body-simulating medium, showing the characteristics of bone-bioactive materials. Osteoblastic cells were observed to anchor to and spread well over the surface of the porous microspheres, and further to proliferate actively with culturing time. The bioactive and porous microspheres developed are considered potentially useful in the regeneration of hard tissues as a matrix for tissue engineering as well as a direct filling material.

Keywords: Bioactive ceramics; Microspheres; Porous structure; Osteoblast culturing; Tissue engineering

1. Introduction

Skeletal defects caused by trauma, damage and developmental recession are generally treated by a variety of therapeutic approaches and with the aid of medical materials [1,2]. Some types of bioceramics, such as calcium phosphates and silica-based glasses, are already used clinically to regenerate defective bone and tooth structures [2–5]. However, their granular forms ranging in size from tens to hundreds of micrometers have been applied mainly to recover the small-sized and/or non-load-bearing defects, owing to mechanical weakness [6]. Granular bioceramics introduced to fill the defective sites act as a scaffold for the cellular population and matrix synthesis, which ultimately form neo-bones and regenerate the structure through a remodeling process [6–8]. Recent studies on bioceramic granules have suggested their advanced use as bioactive fillers in conjunction with polymeric matrices as an injectable device [9,10]. Moreover, these materials might find future applications in the stem-cell-based tissue engineering of bioactive carriers [11,12].

The present study produced porous spherical microparticulates of a bioactive ceramic in an attempt to gain optimal performance of such applications in regenerative medicine. Compared with conventionally used irregular granules with a dense form, the current spherical-shaped microparticles contained highly interconnected pore channels, which are believed to play effective roles in the recruitment of cellular reaction and population and neo-tissue formation.

Based on studies on polymeric or composite microparticles, special emphasis should be paid to control over the macro-structure and composition of the substrate to

* Corresponding author. Address: Department of Biomaterials Science, School of Dentistry, Dankook University, Cheonan, 330-714, South Korea. Tel.: +82 41 550 1926/1928.
E-mail address: kimhw@dku.edu (H.-W. Kim).

1742-7061/$ - see front matter © 2008 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2008.12.006

improve the cell and tissue responses [10–12]. The incorporation of a bioactive inorganic composition, such as bioactive glass and hydroxyapatite within polymeric microspheres resulted in an enhanced osteogenic potential [10,13,14]. Moreover, the introduction of an open space within the polymeric microparticulate was suggested to hold and populate more cells [14,15]. With this in mind, the present study developed porous bioactive ceramic microparticulates, where a novel porogen "Camphene" was used to create open channels. This paper describes the processing tools used to produce porous bioceramic microspheres, along with their in vitro biological performance.

2. Materials and methods

2.1. Preparation of porous microspheres

Calcium silicate powders were first synthesized as a precursor for bioactive ceramic microspheres. Briefly, $Ca(NO_3)_2$ $4H_2O$ and tetraethyl orthosilicate (TEOS) were mixed in ethanol at a molar ratio of 1:1, using a basic catalyst (NaOH). After vigorous stirring, the solution was left to stand at 40 °C until it became a gel, and was then dried at 70 °C. The dried powder was crushed and calcined at 500 °C for 2 h to obtain fine powder.

The calcined powder was mixed with Camphene ($C_{10}H_{16}$, Sigma–Aldrich) which was used as a porogen at 1:8 by weight at a temperature of 50 °C by ball milling, and the slurry mixture was added with a surfactant KD4 (oligomeric hypermer from Uniqema) at 0.75 wt.%. Poly vinyl butyral (PVB, Sigma–Aldrich) dissolved at 10 wt.% in dichloromethane was added at 15 vol.% to the slurry mixture. The use of Camphene in combination with KD4 was based on a previous report [16], and PVB was used as the binder. The mixture was homogenized by ball milling further for 24 h. The slurry mixture was added dropwise into a distilled water pool containing 2% PVA, with continuous stirring at 750 rpm. The slurry mixture was solidified for 30 min, and washed with ice-cooled distilled water through a filter paper. The filtered product was stored at −20 °C for 10 min, gathered onto an alumina crucible and dried overnight under ambient conditions. The weight change of the microspheres due to the sublimation of Camphene during the drying process was measured. The dried product was heat treated as follows: ramping to a temperature of 1400 °C at a heating rate of 2 °C min^{-1}, holding for 3 h, and air cooling in a furnace.

The experimental procedures used to prepare the porous bioactive microspheres are shown schematically in Fig. 1.

2.2. Characterization

The morphology and microstructure of the microspheres were evaluated by scanning electron microscopy (SEM; Hitachi) after the Pt coating. The size distribution of the microspheres was analyzed from the images using optical microscopy. The phase of the powders either heat-treated or not was analyzed by X-ray diffraction (XRD) using Cu $K_{\alpha 1}$ radiation ($\lambda = 1.54056$ Å) at a scanning rate of 2 min^{-1}.

The in vitro bioactivity of the microspheres was assessed by incubating the microspheres in a simulated body fluid (SBF; containing ions of Na^+ 142.0 mM, K^+ 5.0 mM, Ca^{2+} 2.5 mM, Mg^{2+} 1.5 mM, Cl^- 147.8 mM, HCO_3^- 4.2 mM, HPO_4^{2-} 1.0 mM, and SO_4^{2-} 0.5 mM) [17]. In particular, 100 mg of the microspheres contained in the polyethylene tube was immersed in 10 ml of SBF and incubated at 37 °C for different periods, with continuous stirring at 120 rpm. After incubation, the samples were removed, washed with distilled water and ethanol, dried in the oven, and the change in weight was recorded. The surface morphology was examined by SEM to observe precipitates produced on the microsphere surface, and the elements of the precipitates were analyzed by energy dispersive spectroscopy (EDS).

2.3. Cell growth test

Preliminary cellular tests on the porous microspheres were carried out using undifferentiated murine calvarial cells (MC3T3-E1). Before the cells were seeded, the microspheres were washed twice with serum-free medium and loaded into the individual wells of a 96-well plate. Ten milligrams of the microspheres was placed into each well of a 96-well plate, and then a 75 μl aliquot of the cell suspension prepared at a density of either 6×10^4 cells ml^{-1} (low density) or 5×10^5 cells ml^{-1} (high density) was dropped onto each well containing the microspheres. After culturing for 6 h in an incubator humidified with 95% CO_2, 125 μl aliquot of a culturing medium supplemented with 50 μg ml^{-1} sodium ascorbate, 10 mM sodium β-glycerol phosphate and 10 nM dexamethasone was added to each well in order to allow osteoblastic differentiation.

After culturing for pre-determined periods, the morphology of the cells grown on the microspheres was observed by SEM after being fixed with glutaraldehyde, dehydrated with a graded series of ethanol (50%, 70%, 90%, 95% and 100%), treated twice with a hexamethyldisilazane (HMDS) solution, and coated with Pt.

The level of cell growth was measured using an MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium) assay. At each culturing period (1, 5 and 15 day), the culture medium was decanted, and the CellTitero 96 AQueous One Solution Reagent (Promega, Madison) was added to each sample and reacted at 37 °C for 2 h. The absorbance at 490 nm was read using an Elisa Plate Reader.

Five replicate samples were tested for each condition, and the data were expressed as means ± SD. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Bonferroni correction. The P value was considered significant at a level <0.05.

ID	Title	Pages
1329	Preparation of porous bioactive ceramic microspheres and in vitro osteoblastic culturing for tissue engineering application	7