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Mechanical and cytotoxicity evaluation of nanostructured hydroxyapatite-bredigite scaffolds for bone regeneration

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ABSTRACT

Despite the attractive characteristics of three-dimensional pure hydroxyapatite (HA) scaffolds, due to their weak mechanical properties, researches have focused on the development of composite scaffolds via introducing suitable secondary components. The aim of this study was to develop, for the first time, three-dimensional HA-bredigite ($Ca_7MgSi_4O_{16}$) scaffolds containing various amounts of bredigite nanopowder (0, 5, 10 and 15 wt.%) using space holder technique. Transmission electron microscopy, scanning electron microscopy, energy-dispersive X-ray spectroscopy and X-ray diffraction spectroscopy were applied in order to study the morphology, fracture surface and phase compositions of nanopowders and scaffolds. Furthermore, the effects of scaffold composition on the mechanical properties, bioactivity, biodegradability, and cytotoxicity were also evaluated. Results showed that the composite scaffolds with average pore size in the range of 220–310 μm, appearance porosity of 63.1–75.9% and appearance density of 1.1 ± 0.04 g/cm^3 were successfully developed, depending on bredigite content. Indeed, the micropore size of the scaffolds reduced with increasing bredigite content confirming that the sinterability of the scaffolds was improved. Furthermore, the compression strength and modulus of the scaffolds significantly enhanced via incorporation of bredigite content from 0 to 15 wt.%. The composite scaffolds revealed superior bioactivity and biodegradability with increasing bredigite content. Moreover, MTT assay confirmed that HA-15 wt.% bredigite scaffold significantly promoted cell proliferation compared to tissue culture plate (control) and HA scaffold. Based on these results, three-dimensional HA-bredigite scaffolds could be promising replacements for HA scaffolds in bone regeneration.

1. Introduction

Porous calcium phosphate bioceramics have attracted wide attention for bone tissue engineering (BTE) applications due to their interconnected pores affording a promising environment for bone ingrowth and regeneration [1]. Hydroxyapatite, $Ca_{10}(PO4)_6(OH)_2$, referred as HA, is a calcium phosphate bioceramic similar to the mineralized component of bone tissue [2]. In the recent years, porous HA scaffold has been widely investigated to improve attachment, growth and infiltration of osteoblasts compared to dense HA constructs [3]. The porous HA scaffold also shows better osseointegration with host bone tissue than dense one due to interlocking potential [4].

A number of fabrication techniques has been applied for constructing porous scaffolds consisting of camphene-based freeze casting [5], gel casting [6], polymer sponge [7] and space holder [8]. Due to the excellent controllability over the pore size, shape and interconnectivity as well as mechanical strength [8], the space holder technique is well-known as a promising technique for scaffold fabrication. In this technique, the porosity and pore size of scaffolds could be easily controlled using different templates (such as carbamide particles [9] and magnesium particles [10]) acting as porogens within the scaffolds. This technique has been widely applied for development of metallic scaffolds [8]. The space holder technique has recently been applied for the development of porous HA scaffolds using progenies such as PVA fibers and NaCl crystals [3]. The scaffolds consisted of interconnected porosities with pore sizes in the range of 250–400 μm making it suitable for bone regeneration [3].

Despite the excellent biocompatibility, the applications of porous HA scaffolds in the load-bearing situations have been restricted due to its weak fracture toughness and brittle nature [11–13]. A well-known technique to improve the mechanical characteristics of HA scaffold is the development of HA matrix composite scaffolds [14,15]. For instance, porous HA-zirconia scaffold has been recently developed using sponge foam and results demonstrated a significant improved mechanical properties compared to pure HA scaffold [16]. Recently, magnesium-containing silicate ceramics have been introduced as promising candidates for BTE applications due to their good bioactivity, biocompatibility and better mechanical properties than HA [17–19]. Results confirmed that the release of specific ions (such as Mg and Si) from these

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bioceramics could stimulate bone regeneration [20]. Bredigite with chemical formula $Ca_7MgSi_4O_{16}$ is a member of this bioactive ceramic group. Due to good bioactivity, biodegradability and mechanical properties, bredigite has recently been utilized for BTE [21,22]. For instance, pure bredigite scaffold with 90% porosity, large pore size (300–500 μm), good degradation rate and acceptable mechanical properties (compressive strength = 233 ± 14 kPa) was prepared using a polymer sponge approach [21]. However, according to our knowledge, porous HA- bredigite scaffolds have not been investigated, yet.

The aim of this study was to fabricate and characterize three-dimensional HA-bredigite scaffolds. After synthesize HA and bredigite nanopowders through sol-gel technique, composite scaffolds containing various amounts of bredigite nanopowder were developed using space holder technique. Finally, the effects of bredigite content on the physical and mechanical properties as well as bioactivity, biodegradability and *in vitro* cytotoxicity of the scaffolds were investigated.

2. Materials and methods

2.1. Synthesize of HA and bredigite nanopowders

HA nanopowder was synthesized using sol-gel method, based on the previous protocol [23]. Briefly, specific amount of phosphorus pentoxide (P_2O_5, Merck, Germany) was dissolved in absolute ethanol (Merck, Germany) to form a 0.5 mol/l solution. An specific amount of calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$, Merck, Germany) was separately dissolved in absolute ethanol (Merck, Germany) to form a 0.5 mol/l solution. As prepared solutions mixed in a Ca/P molar ratio of 1.67, was aged for 24 h at room temperature to get a transparent gel. The obtained gel was dried at 80 °C in an electrical air oven. The dried gel was heated at a rate of 5 °C/min up to 600 °C, kept for 20 min at this temperature and then cooled at room temperature.

Bredigite powder was also synthesized by a simple combustion method based on citric-nitrate technique [24]. The starting materials were calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$, Merck, Germany), magnesium nitrate hexahydrate ($Mg(NO_3)_2.6H_2O$, Merck, Germany), colloidal silica (SiO_2, Merck, Germany), ammonium nitrate (NH_4NO_3, Merck, Germany) and citric acid ($C_6H_8O_7.H_2O$, Merck, Germany). All the reagents were sequentially dissolved in deionized water to obtain a transparent solution. The solution was dehydrated at 80 °C to form a sol, followed by a further heating at 150 °C to remove excess water. The dried gel was calcined at 1000 °C for 4 h.

2.2. Fabrication of nanocomposite HA-bredigite scaffolds

HA nanopowder was mixed homogeneously with 0, 5, 10 and 15 wt.% bredigite nanopowder using high energy ball milling (Restsch PM100, Germany) in a zirconia cup for 15 min. Three-dimensional scaffolds were produced using space holder technique. In this technique, sodium chloride (NaCl, Merck, Germany) with particle size of 300–420 μm was applied as the space holder. After mixing NaCl particles and powders in weight ratio of 4:1, polyvinyl alcohol (PVA, Sigma) solution (5 wt.%) was added to the above mixture. The mixture was uniaxial pressed in 200 MPa to prepare green sample with diameter of 12 mm. The green samples were heated up to 600 °C, and then, to 1100 °C at a heating rate of 4 °C/min to remove PVA and NaCl particles and create pores. The samples were subsequently sintered at 1350 °C for 2 h.

2.3. Characterization of powders and scaffolds

2.3.1. Structural and physical characterization

Phase structure analysis was carried out by X-ray diffractometer (XRD, Philips xpert) using Ni filtered Cu Kα (λ CuKα = 0.154186 nm, radiation at 40 kV and 30 mA) over 2θ range of 20–80° (time per step: 2.5 s and step size: 0.05°). The average crystallite size was determined for calcined powder and the sintered scaffolds from broadening of XRD peaks using Scherrer equation (Eq. (1)) and SigmaPlot software [25]:

$$Xs = \frac{K.\lambda}{\beta.Cos\,\theta} \tag{1}$$

where K is the shape factor (around 0.9), λ is the X-ray wavelength (nm), β is the line broadening at half of the maximum intensity (FWHM) in radians, θ is the Bragg angle in degree and X_s is the size of the crystalline domains in nm. In this way, three diffraction peaks of HA ((130), (002), and (400) planes) were selected in XRD patterns to determine the crystallite size. The crystallinity of HA nanopowder was determined using Eq. 2 [26]:

$$Xc = 1 - \frac{(V_{112/300})}{I_{300}} \tag{2}$$

where I_{300} is the intensity of (300) diffracted plane and $V_{112/300}$ is the intensity of the hollow between (112) and (300) diffracted planes of HA.

The quantity values of the phases in the sintered scaffolds were calculated based on the following equation (Eq. (3)) [27]:

$$I_{eij} = \frac{K_{ei}X_{ij}}{\rho_i\mu_j^*} \tag{3}$$

where I_{eij} is the intensity of the diffraction peaks from a set of crystallography planes in sample j, analyzed by XRD. K_{ei} is a constant which depends on the nature of phase and it is different for each peak. ρ_i is the density of phase I, X_{ij} is the weight percent of phase I in sample j and μ_j^* is the mass absorption coefficient of sample j.

Transmission electron microscope (TEM, Philips, 208S 100KV) was also applied to study the morphology and particle size of the synthesized HA powder. The morphology and distribution of elements (Ca, P, Mg, Si) in the synthesized powders and sintered scaffolds were evaluated using scanning electron microscope (SEM, Phillips XL 30: Eindhoven, Netherlands) coupled with an energy-dispersive spectroscope (EDS). The scaffolds were sputter-coated with a thin layer of gold and the SEM images were utilized to determine the average pore and grain sizes of sintered scaffolds ($n = 5$) using (NIH) Image J software. The density and porosity of the sintered scaffolds were measured using water displacement method (Archimedes) (Eqs. (4)-(6)) [28].

$$\text{True density} = \text{wt.}\%_{HA}.\rho_{HA} + \text{wt.}\%_{\text{bredigite}}.\rho_{\text{bredigite}} \tag{4}$$

$$\text{Appearance density} = W_a/(W_b - W_c) \tag{5}$$

$$\textit{Appearanceporosity} = (W_b - W_a)/(W_b - W_c) \tag{6}$$

where $\text{wt.}\%_{HA}$ and $\text{wt.}\%_{\text{bredigite}}$ are the weight percent of HA and bredigite, respectively, ρ_{HA} and $\rho_{\text{bredigite}}$ are the density of HA and bredigite, respectively, W_a is dry weight, W_b is the weight of the samples with absorbed water, and W_c is the weight of the samples soaked in water.

2.3.2. Mechanical characterization

Compressive strength and modulus were estimated using a compression test machine (Hounsfield, H25KS) at room temperature, on three specimens (with 12 mm diameter and 24 mm length) for each group at a speed of 1 mm/min. Compressive modulus was calculated via the slope of the initial linear portion of the stress–strain graphs.

2.3.3. In vitro bioactivity evaluation

In vitro bioactivity was studied by soaking the scaffolds in simulated body fluid (SBF) for 28 days. The SBF solution was prepared as described by Bohner [29]. The scaffolds were immersed in SBF solution with the solid/liquid ratio of 10 mg/ml in polyethylene bottles and kept in a water bath at 36.5 ± 0.5 °C. The SEM and EDS were applied to evaluate