Preparation and characterization of nano-sized hydroxyapatite/alginate/chitosan composite scaffolds for bone tissue engineering

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Hye-Lee Kim [a], Gil-Yong Jung [b], Jun-Ho Yoon [a], Jung-Suk Han [b,c], Yoon-Jeong Park [b,d], Do-Gyoon Kim [e], Miqin Zhang [f], Dae-Joon Kim [a,*]

[a] Department of Advanced Materials Engineering, Sejong University, 209 Neungdong-ro, Gwangjin-gu, Seoul 143-747, South Korea
[b] Interdisciplinary Program for Bioengineering, Seoul National University, Seoul 152-742, South Korea
[c] Department of Prosthodontics and Dental Research Center, School of Dentistry, Seoul National University, 101 Daehak-no, Jongno-gu, Seoul 110-749, South Korea
[d] Department of Craniomaxillofacial Reconstructive Science and Dental Research Institute, School of Dentistry, Seoul National University, 101 Daehak-no, Jongno-gu, Seoul 110-749, South Korea
[e] Division of Orthodontics, College of Dentistry, The Ohio State University, Columbus, OH 43210, USA
[f] Department of Materials Science & Engineering, University of Washington, Seattle, 302 L Roberts Hall, WA 98195-2120, USA

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ABSTRACT

The aim of this study was to develop chitosan composite scaffolds with high strength and controlled pore structures by homogenously dispersed nano-sized hydroxyapatite (nano-HAp) powders. In the fabrication of composite scaffolds, nano-HAp powders distributed in an alginate (AG) solution with a pH higher than 10 were mixed with a chitosan (CS) solution and then freeze dried. While the HAp content increased up to 70 wt.%, the compressive strength and the elastic modulus of the composite scaffolds significantly increased from 0.27 MPa and 4.42 MPa to 0.68 MPa and 13.35 MPa, respectively. Higher content of the HAp also helped develop more differentiation and mineralization of the MC3T3-E1 cells on the composite scaffolds. The uniform pore structure and the excellent mechanical properties of the HAp/CS composite scaffolds likely resulted from the use of the AG solution at pH 10 as a dispersant for the nano-HAp powders.

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1. Introduction

Scaffolds for bone tissue engineering, which are a promising approach for the treatment of defective and lost bone, are required to have osteoconductivity and biodegradability in addition to their three dimensional (3D) interconnected porous networks [1]. It has been indicated that a scaffold pore size in the range of 100–400 μm is favorable for cell colonization, proliferation and penetration [2,3].

Chitosan (CS), a deacetylated derivative of chitin, has long been considered one of the most attractive natural biopolymer matrices for tissue engineering because of its biocompatibility, biodegradability and structural similarity to glycosaminoglycans [4–6]. Nevertheless, the use of CS has been limited to non-load-bearing applications due to its poor mechanical properties [7]. Unlike numerous other reinforcing materials, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$: HAp) significantly improves the mechanical properties of CS scaffolds because it has a crystal structure similar to that of the inorganic component of natural bone [8–10]. In particular, nano-sized HAp (nano-HAp) morphologically resembles biological apatite [11] such that scaffolds composed of HAp and CS (HAp/CS composite scaffolds) have enhanced osteoconductive, osteoinductive and osteogenic properties [10].

HAp/CS composite scaffolds have been fabricated by several methods, such as the mechanical mixing of HAp and CS [12,13], the *in situ* co-precipitation of HAp [9,14] and the *in situ* precipitation of HAp into CS [15]. The HAp in the HAp/CS composite scaffolds that were prepared by mechanical mixing of constituents were aggregated and formed large clusters in the scaffolds because of the partial dissolution of nano-HAp particles in the acidic CS solution [12]. In contrast, the preparation of HAp/CS composites by the *in situ* precipitation of calcium phosphate led to an uncontrollable Ca/P ratio and crystallinity of HAp [16]. It has also been reported that the pore structure of HAp/CS composite scaffolds collapsed as the HAp content increased higher than 30 wt.% due to the agglomeration of HAp particles [13]. When HAp particles in the HAp/CS composite scaffolds were prepared by mechanical mixing, a more extreme heterogeneity of pore structure and low compressive strength were observed because the homogeneous dispersion of nano-HAp within the scaffolds was difficult to accomplish due to particle agglomeration [5]. Thus, uniform dispersion of nano-HAp in the composite scaffolds is a prerequisite to produce controlled pore structure and strength of scaffolds.

* Corresponding author.
E-mail address: djkim@sejong.ac.kr (D.-J. Kim).

It has been reported that alginate (AG), which is derived primarily from brown seaweed and bacteria, forms stable hydrogels through the ionic interaction between the carboxyl group of AG and Ca^{2+} [17]. Teng et al. reported that AG promoted the preferential synthesis of HAp oriented in the c direction when composite scaffolds were fabricated by the co-precipitation method [18]. The results indicated a strong ionic interaction between AG and Ca^{2+}, leading to the formation of a specific stereo-chemical arrangement and charge distribution [18]. Furthermore, the combination of AG and CS forms a polyelectrolyte complex (PEC) between the carboxyl groups of AG and protonated amines of CS [19,20]. The formation of a PEC alleviates the shortcomings of CS, such as its poor mechanical properties and structural instability after transplantation [19,20]. Thus, it is expected that AG interacts with both HAp and CS in HAp/CS composites. In this context, AG may be utilized as a dispersant of nano-HAp particles in CS solution since biocompatibility of commercial dispersants for inorganic powders have not been ensured. To the best knowledge of the present authors, there has been no attempt to apply AG as a dispersant for optimization of the pore structure and the mechanical properties of HAp/CS composites.

In this study, nano-HAp/AG/CS composite scaffolds were fabricated utilizing AG as a dispersing agent for nano-HAp particles and freeze-drying the solution mixtures of HAp, AG and CS. The pore morphologies and mechanical properties of the composite scaffolds were determined, and osteoblast differentiation and mineralization on the composite scaffolds were investigated to evaluate the bioactivity and osteoconductivity of the composite scaffolds.

2. Materials and methods

Nano-HAp was hydrothermally synthesized using 1 M $Ca(NO_3)_2 \cdot 4H_2O$ (Sigma-Aldrich, St. Louis, MO, USA) and 1 M H_3PO_4 (Sigma-Aldrich). The solutions were mixed to achieve the Ca/P ratio of 1.67. Then, NH_3 (30%, Sigma-Aldrich) was added into the solution mixture to precipitate the hydroxide containing Ca^{2+} and PO_4^{3-}. The coprecipitates were aged for 24 h at 80 °C and were then placed in an autoclave for 2 h at 180 °C. The hydrothermally synthesized powders were washed three times with deionized water to remove any unreacted substances.

A 4 wt.% CS (practical grade, >75% deacetylated, M_W = 190 kDa–375 kDa, Sigma-Aldrich) solution was prepared by dissolving the CS in deionized water with 1 wt.% acetic acid and the pH of CS solution was 5.2. In addition, 2 and 3.75 wt.% AG (alginic acid from brown seaweed, MW = 80 kDa–120 kDa, Sigma-Aldrich) solutions were prepared for the dispersion of nano-HAp particles and to form a PEC with CS, respectively, by dissolving the AG in deionized water. Prior to the dispersion of the nano-HAp powder, the pH of the 2 wt.% AG solution was adjusted to 9.5–10.0 by adding 5 wt.% NH_4OH solution. During the dispersion of the nano-HAp powder in the 2 wt.% AG solution the pH of the slurry was maintained at about 9.0 using the NH_4OH solution. The dispersion was facilitated by a magnetic stirrer for 1 h and a subsequent sonication for 2 h. The dispersed nano-HAp slurry was mixed with the 3.75 wt.% AG solution using a planetary mixer (ARM-300, Thinky, Laguna Hills, CA, USA) at 2000 rpm for 5 min. HAp/AG/CS composite scaffolds were prepared by mixing the HAp/AG mixture with the 4 wt.% CS solution using the planetary mixer. The nano-HAp content of the composite scaffolds ranged from 10 to 70 wt.%. The pH of HAp/AG/CS solutions was 6.3. Following mixing, the mixture was cast in a 24-well plate and refrigerated at 4 °C for 12 h. During the refrigeration, the plate was covered with paraffin film to prevent water from evaporating. The plate was frozen at −20 °C overnight and then lyophilized for 24 h with a freeze drier (Labconco FreeZone 6Plus Freeze Drier, Labconco, Kansas, MO, USA) under vacuum with the collector temperature set at −89 °C to sublimate the ice from the composite scaffolds. Each composite scaffold was crosslinked by 0.2 M $CaCl_2$ solution for 15 min and was then washed three times with deionized water and immersed in deionized
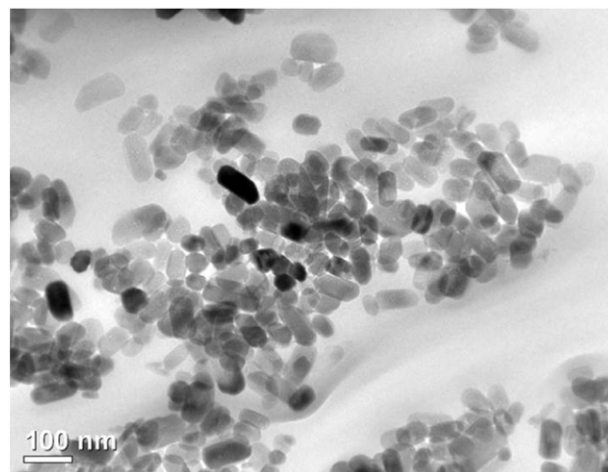

Fig. 1. Transmission electron microscopic images of the nano-HAp particles.

water to remove unbound $CaCl_2$. Wet composite scaffolds were refrozen and lyophilized following the procedure described above.

The prepared nano-HAp, their morphology and particle size were examined using transmission electron microscopy (TEM). This was performed by a JEOL 2010 (Tokyo, Japan), using an accelerating voltage of 200 kV and a copper electron microscopic grid supported by a porous carbon (mesh size 300) film. The zeta potentials of nano-HAp particles suspended in deionized water were measured using electrophoretic light scattering (Otsuka Electronics, Hirakata, Osaka, Japan). The infrared spectra of the composite scaffolds were obtained by Fourier transform infrared (FT-IR) attenuated total reflection spectroscopy (Thermo Fisher Scientific Inc., Franklin, MA, USA). The porosities of the composite scaffolds were measured using the liquid displacement method [21] and calculated by:

$$Porosity(\%) = \frac{(W_1 - W_0)}{\rho \times V_0} \times 100$$

where W_1, W_0, ρ and V_0 are the weight of the composite scaffolds saturated with ethanol, the dry weight of the scaffolds, the density of the ethanol and the initial volume of the composite scaffolds, respectively. Morphometric analysis of composite scaffolds was carried out using microcomputed tomography (micro-CT, Skyscan, Aartselaar, Belgium). The microstructures of cross-sections of the composite scaffolds were observed by scanning electron microscopy (SEM, JEOL, Tokyo, Japan). The mechanical properties of composite scaffolds were determined for circular disk specimens (12 mmØ × 10 mm, n = 5) under compression using a universal testing machine (Instron, Norwood, MA, USA) at a crosshead speed of 1 mm/min until the composite scaffolds reached a 30% reduction
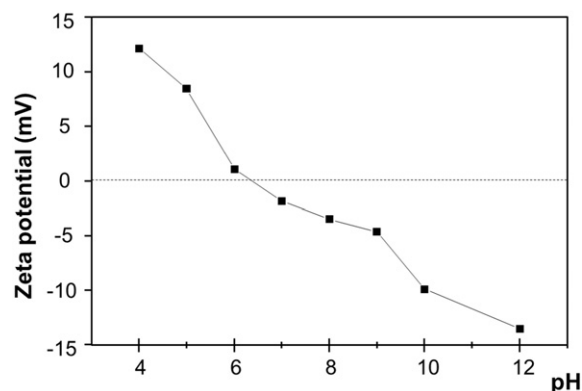

Fig. 2. Effect of pH on the zeta potentials of the nano-HAp particles.