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Short communication

In situ formation of nanostructured calcium phosphate coatings on porous hydroxyapatite scaffolds using a hydrothermal method and the effect on mesenchymal stem cell behavior

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ABSTRACT

The micro/nano-structure of calcium phosphate has been demonstrated to play an essential role in determining cell behavior. However, it is difficult to fabricate micro/nano-structures on the surface of hydroxyapatite (HA) scaffolds. In this study, we have developed a new hydrothermal method for fabricating nanostructures on HA scaffolds with the assistance of 1,2,3,4,5,6-cyclohexanehexacarboxylic acid (H_6L). We controlled the nanostructures of the calcium phosphate coatings deposited on the scaffolds by modulating the H_6L concentration. The results demonstrated that with an increase in the H_6L concentration, the calcium phosphate coatings gradually changed from plate-like, to wire-like, and further to spherical morphology. *In vitro* mesenchymal stem cell (MSC) culture indicated that cell differentiation was significantly enhanced when cultured on the spherical nanostructured coating compared with culture on a plate-like or wire-like nanostructure. Cell proliferation on coatings with different nanostructures was not statistically different with prolonged culture time. These findings indicate that different scaffold surface characteristics, including surface morphology and chemical properties, result in a significant difference in cell differentiation. The small molecule-assisted hydrothermal deposition method provides a simple and controllable route to engineer the nanostructure of HA scaffolds and to construct micro-environments for bone tissue engineering.

1. Introduction

In bone tissue engineering, three dimensional scaffolds with high porosity are required for cell migration, angiogenesis and bone ingrowth [1,2]. To achieve these functions, scaffolds should meet certain criteria, such as mechanical properties, chemical composition, degradability rate and surface topography. These features determine the biological molecule adsorption and the subsequent cell proliferation and differentiation behaviors [3]. Therefore, mimicking bone structure is a good choice for scaffold design. Bone has a complicated hierarchical structure consisting of a nanostructured array of carbonate-apatite crystals aligned within self-assembled collagen fibrils. Furthermore, cancellous bone and cortical bone make up an open macro-porous network [4]. Consequently, a successful scaffold design should consider the characteristics from the macro-scale down to the nano-scale to ensure bone osteogenesis and angiogenesis.

Hydroxyapatite (HA) is chemically similar to the inorganic component of human bones and has excellent biological affinity with bone tissue. Therefore, HA has been used for bone regeneration and is a good candidate for fabricating bone tissue engineering scaffolds [5]. Many studies have focused on the physical characteristics of HA scaffolds, such as pore size and porosity [1,6,7]. Studies have found that macro-pores $>100\ \mu m$ are required for cell migration and nutrient transport and micro-pores $<10\ \mu m$ allow capillary formation and favorable cell-matrix interactions [1,8]. However, few systematic studies have focused on the fabrication of hierarchical micro/nano-structures on pure HA scaffolds and its effect on cell behaviors. Previous studies have demonstrated that the micro/nano-structure

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surface could regulate cell attachment, proliferation and differentiation on HA [9,10]. Human mesenchymal stem cells (hMSCs) cultured on calcium phosphate with a micro-spherical surface morphology formed a greater cellular density than those grown on a plate-like surface [11]. The activity of L929 fibroblast cells cultured on HA nanofibers with a diameter of 50–100 nm was enhanced, whereas cells cultured on nano-crystals < 30 nm were inhibited [10]. The presence of HA nano-needles and nano-fibers limited the activity of osteoblast-like cells, whereas wide nanosheets steadily promoted their proliferation [12]. Taken together, fabricating HA scaffolds with hierarchical micro/nano-structures appears to be an efficient way to manipulate scaffold biological properties while maintaining open and intact macro-pores.

Nanostructured HA (*e.g.*, nanoflakes, nanoclusters, nanospheres) can be deposited on various substrates, such as metals, polymer films or polymer/ceramic composite scaffolds, using bottom-up and top-down technologies, such as electrochemical deposition [13,14], spray coating [15], hydrothermal deposition [16], and biomimetic [11,17]. Nevertheless, little research has addressed modulating the mineral micro/nano-structure that is deposited on the surface of HA scaffolds. The conventional approach to make nanostructured HA scaffold involves directly making synthesized nanostructured HA powders into scaffolds [18] or entrapping HA particles with different shapes within a polymer matrix and then coating the calcium phosphate scaffold surface [6]. These methods require the synthesis of nanostructured HA powders before fabricating HA scaffolds. The procedure is complicated and cannot maximally preserve the HA nanostructure. Recently, a hydrothermal method was reported to prepare HA whiskers in porous biphase calcium phosphate (BCP) scaffolds [19]. The HA whiskers formation process involves dissolution of the β-TCP phase into calcium and phosphate ions and re-deposition of these ions to form HA whiskers. The process is time-efficient and applicable for substrates with complex geometries. However, this method limits application to calcium phosphate scaffolds having a greater solubility than apatite, which means this method is not suitable for HA scaffolds. Considering the effect of nanostructured bioceramics on cell behavior, this study focused on the fabrication of nanostructures on HA scaffolds using a hydrothermal method. First, porous HA scaffolds were fabricated using a simple fiber accumulation method [20], and then, various micro/nano-structured calcium phosphate minerals were deposited onto the scaffold surface with the assistance of small molecules under hydrothermal conditions. In addition, we investigated the effect of nanostructured HA scaffolds on MSC behavior.

2. Materials and methods

2.1. Preparation of porous HA scaffolds

All chemicals were purchased form Kelong Chemical (Chengdu, Sichuan, China) and used as received. First, HA slurry (without calcination) was synthesized using a wet precipitation process using $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$ as the source materials (see [21] for detailed procedures). Then, the slurry was mixed with a sodium alginate solution (2 wt%) at a ratio of 2 to 1 (HA/alginate solution, w/w) to prepare the composite suspension. The suspension was placed in a syringe and dispensed in a $CaCl_2$ solution (150 mM) with a cylindrical mold to rapidly solidify the deposit. The fibrous deposit was further pressed down to produce a cylindrical scaffold [20] (Φ1×1 cm). Subsequently, the fibrous scaffolds were sintered at 1200 °C for 2 h.

2.2. Hydrothermal growth of the nanostructured calcium phosphate coating on HA scaffolds

To achieve calcium phosphate formation on HA scaffolds, a solution of 1,2,3,4,5,6-cyclohexanehexacarboxylic acid (H_6L, purity: > 97%, TCI Development, Shanghai, China) and $Ca(NO_3)_2{\cdot}4H_2O$ (0.1 M) was prepared in 30 ml water. Then, $Na_2HPO_4{\cdot}12H_2O$ was dissolved in the solution to obtain a molar Ca/P ratio of 1.67. Then, urea was added to the solution to 0.6 M, and the pH was adjusted to 2.3 by adding NaOH or HNO_3. To modulate the nanostructures of the mineral coatings, the concentration of H_6L in the solution was varied from 0 to 50 mM. The final solution was transferred to a Teflon-lined autoclave, and the HA scaffold was submerged in the solution. The autoclave was held in an oven at 150 °C for 5 h. After cooling to room temperature, the sample was removed and rinsed with water and dried in air. According to the H_6L concentration used, the corresponding products are termed HA0, HA0.5, HA1, HA5 and HA50.

2.3. Characterization of nanostructured HA scaffold

The morphology and composition of the mineral coatings were analyzed using scanning electron microscopy (SEM, JEOL JSM7001F) equipped with an energy dispersive X-ray (EDX) spectrometer. Structures were analyzed using X-ray diffraction (XRD, X'PertPro MPD, CuKα, 35 mA, 45 kV) and Fourier transform infrared spectroscopy (FTIR, Nicolet 5700). The concentration of the calcium ions released from the HA scaffolds was measured using atomic absorption spectrometry (AAS, Hitachi Z-5000).

2.4. In vitro cell culture

MSCs were purchased from Sichuan University and cultured in α-minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (37 °C, 5% CO_2-95% humidified air). HA scaffolds, which had been sterilized at 121 °C for 30 min, were placed into 24-well culture plates. Then, the MSCs (passage 4) were seeded onto HA scaffolds (HA0, HA0.5, HA5 and HA50) at a density of 1×10^5 cells/scaffold and cultured in the same media supplemented with 50 nM L-ascorbic acid, 10 mM β-glycerophosphate and 0.1 μM dexamethasone. The culture media was refreshed every 2 days.

The adhesion and spreading of the MSCs on the surface of HA scaffolds were observed by fluorescence staining using 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Sigma). Twenty-four hours after seeding, the scaffolds were rinsed with phosphate buffer solution (PBS) and then fixed in a 4% paraformaldehyde solution for 5 min. The fixed cells were then incubated in PBS containing DAPI (100 ng/ml) for 5 min and were rinsed with PBS to remove excess dye. Fluorescence images were taken using a fluorescence microscope (DMIL, Leica, Germany). The images were analyzed using the Image Pro Plus software (IPP, Media Cybernetics, Gaithersburg, MD, USA).

The proliferation activity of MSCs was determined by Alamar blue assays. At 1, 4 and 7 days, the medium was removed, and 300 μL of Alamar blue solution (10% Alamar blue, 80% media and 10% FBS; v/v) was added to each well and incubated at 37 °C for 3 h. Wells without cells were used as the blank control. Then, 200 μL of supernatant fluid was pipetted into 96-well plate and read at 570 nm (excitation)/600 nm (emission) with an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

The alkaline phosphatase (ALP) activity of the MSCs was measured at preset time points (7 and 14 d) using Quantichrom ALP microplate test kits (BioAssay Systems, USA) according to the manufacturer's instructions. Briefly, the medium was removed, and the scaffolds were rinsed three times with PBS. Then, the MSCs were lysed in 500 μL of 0.5% Triton X-100 and shaken at 37 °C for 30 min; 100 μL of the supernatant were mixed with 100 μL of the ALP assay working solution, incubated for 15 min and stopped using 100 μL of a 2 M NaOH solution. The absorbance at 405 nm was measured. The total protein content was measured using the bicinchoninic acid protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nangjing, China). The relative ALP activity was obtained as the changed optical density values divided by the total protein content.