International Journal of Biological Macromolecules 103 (2017) 385–394

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Degradation behavior and compatibility of micro, nanoHA/chitosan scaffolds with interconnected spherical macropores

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ARTICLE INFO

Article history:
Received 25 October 2016
Received in revised form 27 February 2017
Accepted 29 March 2017
Available online 31 March 2017

Keywords:
MicroHA
NanoHA
CS
MC3T3-E1
Enzymatic degradation

ABSTRACT

Hydroxyapatite/Chitosan (HA/CS) composite have significant application in biomedical especially for bone replacement. Inorganic particle shape and size of composite affect the scaffold mechanical property, biological property, and degradation. The aim of this study was to fabricate HA/CS scaffold with good pore connectivity and analyze their biological, degradation properties. Microhydroxyapatite/chitosan (mHA/CS) and nanohydroxyapatite/chitosan (nHA/CS) composite scaffolds with interconnected spherical pore architectures were fabricated. Composite scaffolds structure parameters were analyzed using micro CT. Cell proliferation and morphology were tested and compared between two scaffolds using mouse osteoblastic cell line MC3T3-E1. To research the composite degradation in lysozyme PBS solution, degradation rate and reducing sugar content were tested, and scaffolds morphology were observed by SEM. The results showed that microHA and nanoHA were fabricated by being calcined and synthesis methods, and their infrared spectra are very similar. EDAX composition analysis demonstrated that both of microHA and nanoHA were calcium deficiency HA. Micro-CT results demonstrated the scaffolds had interconnected spherical pores, and the structure parameters were similar. Cell viabilities were significant increased with cultured time, but there were no significant difference between microHA/CS and nanoHA/CS scaffolds. Scaffold structure was gradually destroyed and inorganic composition HA particles are more prominent with degradation time.

Significance: (1) Inorganic particle shape and size of composite affect the scaffold mechanical property, biological property, and degradation. NanoHA/CS and microHA/CS scaffolds with good pore connectivity were fabricated and their biological, degradation properties were studied in this manuscript. (2) The scaffold with interconnected porosity construct provides the necessary support for cells to proliferate and maintain their differentiated function, and its architecture related to the structure and morphology of new bone. Polymer scaffolds were fabricated by the technique of compression molding and particulate leaching method, and paraffin microspheres were used as the porogen. (3) MicroHA/CS and nanoHA/CS composite scaffolds are potential materials for use in bone tissue engineering.

1. Introduction

The approach of tissue engineering is considered promising to repair or regenerate damaged tissue through the substitution of engineered tissue with the aim that it will help restore the functions during regeneration and subsequent integration with the host tissue [1]. More and more attention is focus on the fabrication of tissue scaffolds, and bone scaffold is an important one in these scaffolds.

Chitosan is a copolymer of glucosamine and *N*-acetylglucosamine derived from the natural polymer chitin, which is commercially available. Chitosan can be hydrolyzed by lysozyme, it is also one of the biodegradable polymers in nature. The degradation rate of chitosan can be controlled and the degraded products of chitosan are nontoxic, nonimunogenic, and

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http://dx.doi.org/10.1016/j.ijbiomac.2017.03.175
0141-8130/

noncarcinogenic [2,3]. Hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$], is the fundamental inorganic component of bone and is a biologically active calciumphosphate ceramic that is employed in surgery to replace and mimic bone [4,5]. The shape of HA crystals in a natural bone is needlelike or rod-like with length and width of 40–60 nm and 10–20 nm, respectively [6,7]. The HA thus formed displayed bone-bonding properties, has been extensively used in hard tissue replacement in view of their biocompatibility and osteoconductivity features, but the brittleness of HA materials limits its application in bone tissue engineering.

As bone tissue repair materials, HA/CS composites have been studied for many years. HA [8] or CS [9] is the matrix material in composite scaffolds. In the HA system, CS was introduced to solve the problem of HA particle formation and the increase of the strength. In CS matrix, HA was added and scattered among CS matrix to improve the bone bond between HA/CS material and bone tissue [10,11].

Many research have shown that a material with a highly porous structure would provide more space for cells to attach and grow so that it has better biological properties [12]. Particulate leaching method provides easy control of pore structure and has been widely employed in the fabrication of scaffolds [13–15]. The scaffold made by the salt leaching method has good compressive strength, but it has some cytotoxicity from the residue salt particles and a poor interconnected porous structure [16]. Zhang et al. [17] reported that scaffolds had better pore connectivity with the spherical pores resulting from paraffin than the cubic pores resulting from inorganic salts.

The resource and size of HA is an important factor on composite scaffolds. Different kind of methods have been used to prepare the HA from natural bone such as thermal calcination, alkaline hydrolysis and subcritical water method [18–20]. Thermal calcination method usually produces mHA, whereas, alkaline hydrolysis method, produces carbonated nHA [18]. Literature suggests that carbonated HA has comparatively higher osteoconduction, bio resorption and biocompatibility with regard to bone formation [21,22] than synthetic HA. Naturally derived mHA combined chitosan has already been investigated for bone formation [23,24]. Li et al. [25] prepared micro and nanoHA/CS films, then studied the surface characters and biocompatibility, the results showed that both microHA/CS films and nanoHA/CS films had excellent biocompatibility, moreover, the MSCs on nanoHA/CS films present higher osteogenic differentiation activity than on microHA/CS films. Also, HA possesses the double functionality of cross-linking agent and inorganic reinforcement. Munarin et al. [26] reported the grain size and specific surface area of the HA powders affected the gelling time and rheological properties of the hydrogels at room temperature. The results pointed out that microHA could be proposed for applications which required rapid gelling kinetics and improved mechanical properties, conversely the nanoHA synthesized in the work seems the best choice to obtain homogeneous hydrogels with more easily controlled gelling kinetics. However, the report about effect of the HA particle size of HA/CS composite materials on the proliferation, morphology of the cell and the enzymatic degradation behavior was less.

In the present study, microHA was prepared from porcine bone by degreasing, deproteinization, calcination and ball milling, and nanoHA was synthesized by ultrasonic and stirring method. Micro, nanoHA/CS composites with interconnected spherical macropores were prepared by blending solution casting, particle exporting using paraffin microspheres as porogen. MC3T3-E1 cells were seeded into the scaffolds, and the biocompatibility of the scaffold was investigated, and the enzymatic hydrolysis of the composite scaffolds in vitro was investigated.

2. Materials and methods

2.1. Materials

CS (200–800 cP, Brookfield (lit.), degree of deacetylation is 75–85%) was purchased from the Sigma Chemical Company and used without further purification. Paraffin with a melting point between 53 °C and 57 °C, gelatin, and N-hexane were obtained from Tianjin Kemiou Chemical Reagent Company.

2.2. MicroHA, nanoHA fabrication and characterization

2.2.1. Micro-HA fabrication

The porcine trabecular bone was collected from nearby butcher shops and all of the attached meat and fat were removed and cleaned from the bones. The ends of the cancellous bone were cut into slices about 2 mm, then cyclic soaked in hydrogen peroxide and ethyl ether to be degreased, deproteined, then being washed and dried. The dried degreased and deproteined bone was calcined (1000 °C, 3 h) to prepare the true bone ceramic (TBC), then the TBC was being ball milled at 230 r/min for 2.5 h.

2.2.2. NanoHA fabrication

NanoHA was synthesized by wet chemical reaction:

$$10Ca(NO_3)_2{\cdot}4H_2O + 6(NH_4)_2HPO_4 + 8NH_3{\cdot}H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 46H_2O$$

$Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ aqueous solution with concentration of 0.5 mol/L were prepared, and pH value was adjusted to 10.2 with ammonia. According to the molar ratio of Ca/P 1.67, $(NH_4)_2HPO_4$ aqueous solution was slowly added to $Ca(NO_3)_2$ aqueous (polyethylene glycol (PEG) as dispersing agent) under ultrasonic, then mechanical stirring for 1 h at room temperature and ammonia was used to regulate solution pH value constant at about 10.6. After static aging for 24 h, the obtained hydrogel was centrifugal separated, washed to neutral using deionized water, and then washed with absolute ethyl alcohol and vacuum filtration. The sample was dried at 50 °C, and calcined at 1000 °C for 3 h to obtain nanoHA.

2.2.3. Characterization

MicroHA and nanoHA samples were studied with the FT-IR spectroscopy. Samples were mixed with pre-dried FT-IR grade KBr and pressed into disks for the study. Transmission infrared spectra of disks were recorded using a FTIR spectrometer (NICOLET380, USA). The FTIR spectra of the samples were obtained in the spectral range from 4000 to 400 cm^{-1}. The Ca, P content of microHA and nanoHA were analysed by energy dispersive X-ray analysis (1530VP, LEO, Germany).

2.3. Preparation of HA/CS composite porous scaffolds [27]

Paraffin microspheres were used as the porogen in the fabrication of the scaffolds. Polymer scaffolds were fabricated by the technique of compression molding and particulate leaching method [17,28].

In brief, HA was dispersed in deionized water by ultrasonic, then CS and acetic acid was added under stirring for 1–2 h at room temperature until a homogeneous solution was obtained. Sieved porogen particles were added into the polymer solution to form a paste-like mixture which was then pressed into a PTFE mould and kept in a vacuum oven for 72 h to remove residual solvents. The composite sample was then taken from the mould and the porogen particles were removed using Soxhlet extractor with N-hexane as