Materials Science and Engineering C 72 (2017) 134–142

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Strontium hydroxyapatite/chitosan nanohybrid scaffolds with enhanced osteoinductivity for bone tissue engineering

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ARTICLE INFO

Article history:
Received 29 August 2016
Received in revised form 3 November 2016
Accepted 9 November 2016
Available online 18 November 2016

Keywords:
Strontium hydroxyapatite
Bone tissue engineering
Osteoinductivity
Porous scaffold

ABSTRACT

For the clinical application of bone tissue engineering with the combination of biomaterials and mesenchymal stem cells (MSCs), bone scaffolds should possess excellent biocompatibility and osteoinductivity to accelerate the repair of bone defects. Herein, strontium hydroxyapatite [SrHAP, $Ca_{10-x}Sr_x(PO_4)_6(OH)_2$]/chitosan (CS) nanohybrid scaffolds were fabricated by a freeze-drying method. The SrHAP nanocrystals with the different *x* values of 0, 1, 5 and 10 are abbreviated to HAP, Sr1HAP, Sr5HAP and Sr10HAP, respectively. With increasing *x* values from 0 to 10, the crystal cell volumes and axial lengths of SrHAP become gradually large because of the greater ion radius of Sr^{2+} than Ca^{2+}, while the crystal sizes of SrHAP decrease from 70.4 nm to 46.7 nm. The SrHAP/CS nanohybrid scaffolds exhibits three-dimensional (3D) interconnected macropores with pore sizes of 100–400 μm, and the SrHAP nanocrystals are uniformly dispersed within the scaffolds. In vitro cell experiments reveal that all the HAP/CS, Sr1HAP/CS, Sr5HAP/CS and Sr10HAP/CS nanohybrid scaffolds possess excellent cytocompatibility with the favorable adhesion, spreading and proliferation of human bone marrow mesenchymal stem cells (hBMSCs). The Sr5HAP nanocrystals in the scaffolds do not affect the adhesion, spreading of hBMSCs, but they contribute remarkably to cell proliferation and osteogenic differentiation. As compared with the HAP/CS nanohybrid scaffold, the released Sr^{2+} ions from the SrHAP/CS nanohybrid scaffolds enhance alkaline phosphatase (ALP) activity, extracellular matrix (ECM) mineralization and osteogenic-related COL-1 and ALP expression levels. Especially, the Sr5HAP/CS nanohybrid scaffolds exhibit the best osteoinductivity among four groups because of the synergetic effect between Ca^{2+} and Sr^{2+} ions. Hence, the Sr5HAP/CS nanohybrid scaffolds with excellent cytocompatibility and osteogenic property have promising application for bone tissue engineering.

1. Introduction

For the reconstruction of bone defects due to trauma or disease, autologous bones have still been used as "gold standard" in orthopaedic surgery [1,2]. However, this methodology requires second invasive surgery, leading to donor site morbidity, nerve damage, inflammation and hematomas [3,4]. Recently, bone tissue engineering has been developed for alternative therapy by the combination of biomaterials, cells or growth factors [5,6]. In order to meet the demand of clinical applications, bone tissue engineering materials should possess three-dimensional (3D) macropores with pore sizes of 100–400 μm to promote the cell adhesion, spreading and tissue ingrowth into the center of implants [7,8]. Moreover, the bone tissue engineering scaffolds should have favorable osteoinductivity in order to accelerate the formation of new bones.

Apatite is one of main inorganic minerals in vertebrate hard tissues such as 60–70% bone and 98% dental enamel, so the corresponding synthetic hydroxyapatite [HAP, $Ca_{10}(PO_4)_6(OH)_2$] based composite scaffolds have been widely used for bone implants [9–12]. The above inorganic-organic composite scaffolds have the advantages of unlimited resource, stable quality, perfect biocompatibility and osteoconductivity, but they lack osteoinductivity. In order to accelerate osteogenic differentiation, two common strategies have been developed by loading cytokines into scaffolds or by doping trace elements in bone scaffolds. The cytokines, like bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are utilized to improve osteogenic ability [13,14]. However, the loading-release properties of cytokines in bone scaffolds are difficult to control because they are determined by scaffold microstructures and environmental conditions such as chemical composition, porous structure, surface characteristic, pH value and temperature [15,16]. The high concentrations of released cytokines may even lead to adverse effects [15,17]. Another alternative strategy is to dope bioactive trace elements into HAP by ion substitution. The therapeutic ions including Sr^{2+},

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http://dx.doi.org/10.1016/j.msec.2016.11.063
0928-4931/

Zn^{2+}, Mg^{2+}, Cu^{2+} and Si^{4+} ions can accelerate angiogenesis and simulate the osteogenic differentiation of mesenchymal stem cells (MSCs) [18–20].

Among the bioactive metal ions, strontium (Sr) ions have been widely investigated in bone repair materials, because they are able to increase new bone formation and inhibit bone resorption [21–24]. One of the mechanisms is that Sr ions can promote the osteogenesis of MSCs and block the proliferation of osteoclasts [23,25]. The oral drugs containing Sr like strontium ranelate (SrR) is a direct way to enter into body, but lack controlled local release property in bone defect sites [26,27]. Fortunately, the above disadvantage can be addressed by substitution of Ca^{2+} ions in HAP by Sr^{2+} ions, since the ionic radius of Sr^{2+} ions (1.12 Å) is similar to that of Ca^{2+} ions (1.00 Å). The strontium hydroxyapatite (SrHAP) not only overcomes the disadvantages of HAP with no osteoinductivity, but also controls the sustained release of Sr^{2+} ions from SrHAP. Marie et al. have reported that Sr^{2+} ions can accelerate bone formation at a low dose, and increase bone mass in animals [21]. However, the effects of Sr^{2+} concentrations on biological properties have been rarely reported up to now.

Natural bone is a hierarchically nanostructured composites with the dispersion of apatite nanocrystals along collagen fibers [28]. The chemical compositions and porous structures of natural bones inspire us to design novel organic-inorganic bone scaffolds [10–12]. Chitosan (CS) derived from natural chitin is a linear polysaccharide copolymer of *N*-acetyl-D-glucosamine and D-glucosamine [29]. It has many advantages for the application in bone tissue engineering, including excellent biocompatibility, intrinsic antibacterial property, appropriate biodegradability and the ability to improve new bone formation [29,30]. In this work, the HAP and collagen in natural bones are replaced by SrHAP and chitosan (CS), respectively. The main aims of this work are to fabricate SrHAP/CS nanohybrid scaffolds, and investigate their structure, morphology and biocompatibility, and discuss the effect of Sr percentages in SrHAP nanocrystals on osteoinductivity.

2. Experimental

2.1. Preparation of SrHAP nanocrystals

The SrHAP nanocrystals with different Ca/Sr ratios were fabricated according to the following steps (Table 1). Firstly, the mixed Ca^{2+} and Sr^{2+} solutions were prepared by the dissolution of $Ca(NO_3)_2$ and $Sr(NO_3)_2$ into 250 ml deionized water. At the same time, the PO_4^{3-} solution (0.30 M) was prepared by the dissolution of $(NH_4)_2HPO_4$ into 250 ml deionized water. The pH values of all solutions were adjusted to 10.0 by $NH_3 \cdot H_2O$ solutions. Secondly, after the temperature increased to 90 °C, the mixed Ca^{2+} and Sr^{2+} solutions were stirred by a mechanical agitation, followed by the slow addition of the $(NH_4)_2HPO_4$ solution within 40 min. The as-obtained mixture was stirred at 90 °C for 1 h, and then aged further for 12 h. Finally, the SrHAP nanocrystals with different Ca/Sr ratios were prepared (Table 1), after the precipitations were washed with deionized water and ethanol, dried at 60 °C and sintered at 900 °C for 4 h.

Table 1
Abbreviations, molecular formulas, Ca/Sr ratios, $Ca(NO_3)_2$ and $Sr(NO_3)_2$ concentrations for the preparation of SrHAP nanocrystals.

Sample	Molecular formula	Ca/Sr ratio	$Ca(NO_3)_2$ concentrations	$Sr(NO_3)_2$ concentrations
HAP	$Ca_{10}(PO_4)_6(OH)_2$	10:0	0.50 M	0
Sr1HAP	$Ca_9Sr_1(PO_4)_6(OH)_2$	9:1	0.45 M	0.05 M
Sr5HAP	$Ca_5Sr_5(PO_4)_6(OH)_2$	5:5	0.25 M	0.25 M
Sr10HAP	$Sr_{10}(PO_4)_6(OH)_2$	0:10	0	0.50 M

2.2. Preparation of SrHAP/CS nanohybrid scaffolds

CS powders (4.0 g) were dissolved into an acetic acid solution (100 ml, 2.0 vol%) to obtain a homogeneous CS solution. SrHAP or HAP nanocrystals (10.0 g) were added in the CS solution (40.0 g), followed by stirring for 10–20 h. The uniform mixture was injected into the mould with a thickness of 2 mm and a diameter of 12 mm, and then was freezed at −20 °C for 5 h. The above solidified mixtures were dried in a freeze-drying machine at −35 ~ −60 °C for 36 h. Finally, the SrHAP/CS nanohybrid scaffolds were treated with NaOH solution (50 ml, 10.0 wt%), washed with deionized water, and dried at 60 °C. The inorganic minerals in the HAP/CS, Sr1HAP/CS, Sr5HAP/CS and Sr10HAP/CS nanohybrid scaffolds were HAP, Sr1HAP, Sr5HAP and Sr10HAP nanocrystals, respectively

2.3. Characterization

The morphologies and element maps of SrHAP/CS nanohybrid scaffolds were characterized by scanning electron microscopy (SEM) and energy-dispersive spectrometry (EDS, S-4800 CamScan; Hitachi, Japan), respectively. X-ray powder diffraction data were detected on a Bruker D8 Advance X-ray diffractometer using CuKα radiation ($\lambda = 1.5406$ Å) within the scanning range of $2\Theta = 20–55°$ at step size of 0.02°. Fourier transform infrared (FTIR) spectrometry (Vector22; Bruker Daltonics, Billerica, USA) was used to analyze the functional groups of SrHAP/CS nanohybrid scaffolds using a KBr pellet technique at room temperature in the range of 400–4000 cm^{-1}. The ion release properties of SrHAP/CS nanohybrid scaffolds were characterized by soaking 0.5 g samples in 50 ml simulated body fluid (SBF). The concentrations of Ca^{2+} and Sr^{2+} ions in SBF were detected at different time points by inductively coupled plasma/optical emission spectrometry (ICP/OES; Perkin Elmer, OPTIMA 3300 DV).

2.4. In vitro cell isolation and expand

This study was performed based on the principles of the Helsinki Declaration. The experimental procedures were approved by the Ethical Review Board of Shanghai Sixth People's Hospital, Shanghai Jiaotong University School of Medicine. The human bone marrow mesenchymal stem cells (hBMSCs) were isolated and cultured according to the previous report [31]. In brief, bone debris and large particles were removed by a 70 mm cell strainer, and the remaining cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO) at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was changed to remove non-adherent cells. The hBMSCs of the fifth to seventh passage were used for the following study.

2.5. Cell viability assay

Cell viability was analyzed by using live/dead cell imaging kit (Invitrogen). Various scaffolds were divided into four groups as HAP/CS, Sr1HAP/CS, Sr5HAP/CS and Sr10HAP/CS, and the HAP/CS group served as the control group. The sterile HAP/CS, Sr1HAP/CS, Sr5HAP/CS and Sr10HAP/CS nanohybrid scaffolds were seeded with 1×10^5 hBMSCs in 24-well culture plates. After 2 days, the Live Green and Dead Red reagents were added at room temperature. The cells were observed using a Leica DMI6000 B with Leica AF6000 software. The cells with green and red fluorescence signal represent live and dead cells, respectively.

2.6. Cell adhesion and spreading

The cell adhesion and spreading were observed by scanning electron microscope (SEM). The hBMSCs were seeded on various scaffolds and incubated for 1 and 3 days. The scaffolds with cells were washed and