Materials Science and Engineering C 33 (2013) 3389–3395

Contents lists available at SciVerse ScienceDirect

Materials Science and Engineering C

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

Preparation, characterization and biological test of 3D-scaffolds based on chitosan, fibroin and hydroxyapatite for bone tissue engineering

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ARTICLE INFO

Article history:
Received 6 November 2012
Received in revised form 9 March 2013
Accepted 10 April 2013
Available online 18 April 2013

Keywords:
Chitosan
Fibroin
Hydroxyapatite
3D-Scaffolds
SaOs-2 cells

ABSTRACT

This work describes the preparation and characterization of porous 3D-scaffolds based on chitosan (CHI), chitosan/silk fibroin (CHI/SF) and chitosan/silk fibroin/hydroxyapatite (CHI/SF/HA) by freeze drying. The biomaterials were characterized by X-ray diffraction, attenuated total reflection Fourier transform infrared spectroscopy, thermogravimetric analysis, differential scanning calorimetry, scanning electron microscopy and energy dispersive spectroscopy. In addition, studies of porosity, pore size, contact angle and biological response of SaOs-2osteoblastic cells were performed. The CHI scaffolds have a porosity of 94.2 ± 0.9%, which is statistically higher than the one presented by CHI/SF/HA scaffolds, 89.7 ± 2.6%. Although all scaffolds were able to promote adhesion, growth and maintenance of osteogenic differentiation of SaOs-2 cells, the new 3D-scaffold based on CHI/SF/HA showed a significantly higher cell growth at 7 days and 21 days and the level of alkaline phosphatase at 14 and 21 days was statistically superior compared to other tested materials.

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1. Introduction

Nearly 7% of orthopedic fractures may develop problems of bone consolidation due to bone defects, impaired fracture healing, or a combination of both. Bone graft biomaterials can be used in this situation to fill empty spaces and offer support, consequently optimizing the biological repair of the defect [1,2].

In this context biomaterials for tissue engineering must exhibit simultaneously suitable mechanical properties and a favorable *in vivo* performance in order to reduce the patient's rehabilitation time [3–5]. Thus both biological and physicochemical properties must be carefully considered during the development of biomaterials for tissue engineering. 3D porous scaffolds are used in tissue engineering strategies to provide an appropriate environment for regeneration of injured areas allowing the organization and development of cells in an environment similar to the original tissue. The study of cell behavior, nature of scaffolds and the occurrence of cell stimulation is fundamental to the implant success [3,4]. A large variety of biomaterials and techniques is used to produce porous scaffolds that can be used in different tissues such as bone tissue. However, these materials must meet five basic requirements for bone tissue engineering: biocompatibility; biodegradability; good mechanical properties; architectural similarity with the treated area; and viable production technology [6–10].

In terms of mechanical properties and architecture, the biomaterial must exhibit characteristics similar to the injured area, helping local vascularization and cell migration within the 3D porous scaffold. Finally, for an optimized production, the material characteristics should be reproducible, presenting clinical and commercial viability, effective cost and short production time [5,6,10]. In order to produce composites that mimic bone's composition and architecture, hydroxyapatite has been mixed with biopolymers such as collagen but other candidates are under consideration in order to lower production costs. The fragility of hydroxyapatite (HA) ceramic can be minimized by incorporation of chitosan (CHI) owing to suitable plastic and adhesive properties, avoiding the possibility of migration of HA particles [11]. Some authors observed that composites based on chitosan were mechanically flexible and easily moldable in desirable shapes [12,13]. On the other hand, the use of silk fibroin (SF) in HA composites can improve stability against fragmentation due to the high affinity of fibroin fibrils for hydroxyapatite clusters. Unfortunately, HA/SF composites cannot meet the requirement for bone substitution due to insufficient formability and flexibility [14]. The association of CHI and SF as a binary organic matrix for HA to prepare scaffolds may be attractive for bone tissue engineering because the resulting material will combine biocompatibility, modeling and flexibility. In

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http://dx.doi.org/10.1016/j.msec.2013.04.026

addition, the production costs of these scaffolds may decrease since these three materials are widely available.

To date, few studies have analyzed the relationship among CHI, SF and HA as composite applicable to bone tissue engineering [13,14]. To our knowledge, no systematic studies were reported on the development of 3D porous scaffolds using the three materials of interest here and the evaluation of their biological properties. Finally, the cross-linking of CHI/SF/HA scaffolds necessary to increase their stability in culture medium is also unexplored. Therefore, the aim of this work was to prepare 3D-scaffolds based on chitosan, fibroin and hydroxyapatite and to study their morphological, physical–chemical and biological properties.

2. Materials and methods

2.1. Production of 3D-scaffolds

Silk fibroin (SF) extracted from *Bombyx mori* cocoon was purchased from Huzhou Xintiansi Bio-tech Co., Ltd. (China) while the chitosan (CHI), derived from crab shell, deacetylation degree 85%, was purchased from Sigma-Aldrich (USA). Non-sintered hydroxyapatite (HA) with particle size < 120 μm was a courtesy from the Brazilian Center for Physics Research—CBPF (Brazil). Initially CHI 2% (w/v) was dissolved in 1% aqueous acetic acid. Different scaffolds were prepared using: group I (CHI)—the parent CHI solution; group II (CHI/SF)—a dispersion of SF (1:1 wt.%) in the CHI solution; and group III (CHI/SF/HA)—a dispersion of SF and HA (1:1:2 wt.%) in the CHI solution. To obtain a good homogenization the suspensions remained under magnetic stirring for one day and afterwards sonicated (Ultrasonic Cleaner Thomton 750USC, Unique, Brazil) for 10 min. Subsequently, this suspension was poured into polytetrafluoroethylene mold (height, 40 mm; diameter, 90 mm), frozen for 24 h at −20 °C and lyophilized (Liotop L108, Liobras, Brazil) for 24 h. The dry samples were removed from the molds and neutralized in a 0.1% (wt.%) NaOH aqueous–ethanolic (8:2 vol.%) solution for 3 h. Afterwards samples were washed for 30 min with ultrapure water (UHQ, Purelab, USA) and cross-linked with 2.5% (w/v) sodium tripolyphosphate solution for 3 h, followed by washing for 30 min with ultrapure water. The crosslinking step was necessary because the degradation rate of chitosan was too fast, as observed in previous tests (data not show). Finally, 3D-scaffolds were obtained after another cycle of freezing and lyophilization.

2.2. Physicochemical characterization

Powder X-ray diffraction (XRD) for raw chitosan, fibroin and HA as well as for the composites were measured in a Shimadzu XRD 6000 (Japan) diffractometer using CuKα radiation (λ = 1.5406Å) with 30 mA, 40 kV, and scanning rate of 3°/min. Infrared spectra were measured in the range 2000–650 cm^{-1} using a Fourier transform infrared spectrometer (FTIR; Perkin Elmer Spectrum 100, USA). The measurements were made using ATR (Attenuated Total Reflectance) accessory. The thermo gravimetric analysis (TGA-581, Mettler-Toledo, Switzerland) was measured in the range between 0 and 1000 °C with a heating rate of 10 °C/min. The differential scanning calorimetry (DSC-822, Mettler-Toledo, Switzerland) was performed from 0 to 600 °C with a heating rate of 10 °C/min. The porosity of the 3D-scaffolds was measured by liquid displacement using hexane since it is suitable to avoid swelling or shrinking of the matrix. The 3D-scaffold was immersed in a known volume (V_1) of hexane in a graduated cylinder for 5 min. The total volume of hexane plus the hexane-impregnated 3D-scaffolds was recorded as V_2. The hexane-impregnated scaffold was then removed from the cylinder and the residual hexane volume was recorded as V_3 [15]. The porosity of the 3D-scaffold was obtained by Eq. (1) (Appendix A).

Pore sizes were measured by mercury intrusion porosimetry at low pressure (Autopore IV, Micromeritics, France). The scaffolds' hydrophilicity was qualitatively determined by measuring the contact angle with distilled water using the DSA-100 goniometer (Krüss, Germany). A single drop of volume 2.5 μL was poured onto the 3D-scaffold. The drop shape on the scaffold surface was measured by the sessile drop approximation. The morphology, topography and microstructure of the 3D-scaffolds, before the sterilization step, were observed by scanning electron microscopy (SEM; Jeol JSM 6460-LV, Japan). Elemental analysis was carried out by energy dispersive X-ray spectroscopy (EDS; Noran System Six EDS-200, USA).

2.3. Cell culture

Human osteosarcoma human cells (SaOs-2; HTB-85-ATCC) were used to evaluate cell viability and differentiation in 3D-scaffolds by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and ALP (alkaline phosphatase) tests, respectively. SaOs-2 cells were cultured in McCoy's Medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (VWR International, USA), 1% L-glutamine and 1% penicillin–streptomycin (Sigma Aldrich, USA). 10^{-8} M of dexamethasone (Sigma-Aldrich, USA) was added to culture media to induce osteoblastic differentiation. A cell density of 5×10^5 cells was inoculated onto each 3D-scaffold, which were previously sterilized using gamma radiations at a dose of 25 kGy, and cultivated for 7, 14 and 21 days to obtain growth and differentiation data. Cell morphology was observed by SEM.

The number of viable cells was assessed based on the MTT (Sigma-Aldrich, USA) reduction into insoluble formazan crystals by the mitochondria of living cells. The measurements were made reading the absorbance at 570 nm. Briefly, 800 μL of acidic isopropanol was added to each well to dissolve the intracellularly formed formazan crystals. The cell number was obtained by a linear correlation between absorbance and cell concentration (from 10^4 up to 10^5 cells/mL).

Cell differentiation was evaluated by ALP activity. The samples were permeabilized with 0.5% Triton X-100 and incubated with a 20 nM p-nitrophenyl phosphate (Sigma-Aldrich, USA) solution, 100 mM diethanolamine, 10 mM $MgCl_2$ (pH 9.5) at 37 °C for 30 min. The enzymatic reaction was ended by the addition of 0.1 M EDTA and 1 M NaOH, and the absorbance was read at 405 nm. ALP activity was calculated from a standard curve (from 0 up to 3200 nmol/L) and the results were expressed in nanomol of p-nitrophenol (reaction product) per cell.

The absorbance data of MTT assay and ALP activity assay were obtained by an ELISA plate reader (Bio-Tech Instruments, USA). All the tests were performed in triplicate. Mean values and standard deviations were submitted to variance analysis (ANOVA) followed by the Tukey test considering significance at 0.05. Before, the normal distribution of all data was tested using Shapiro–Wilk test.

3. Results and discussion

Powder XRD patterns for CHI, SF and HA (Fig. 1A) and for studied 3D-scaffolds (Fig. 1B) were performed to investigate their crystalline degree. A peak at 19.6° (*) was assigned to CHI (see patterns of pure CHI and group I) in agreement to Kim et al. [13]. When the SF is added to the CHI matrix (group II), the composite becomes more amorphous as compared to group I. Moreover, the CHI peak becomes less intense and is overlapped by a peak at 20.6°. This new peak together with another one at 24.3° (**) refers to the β-sheet crystalline structure of the SF. Similar data were observed by Du et al. [16], who found peaks at 20.3° and 24.6° for SF in a SF/HA composite. As pointed out by Choi et al. [17] and Feng et al. [18], the amorphous halo formed in both groups is characteristic of the presence of CHI and SF in these biomaterials. Small peaks at 31.8°, 32.2° and 32.9° (***) corresponding to a pattern of pure HA (JCPDS 9-432) were observed for group III. Besides, the CHI peak increases in group III due to the higher amount of this polymer relative to SF. In general, the CHI and SF did not alter the crystallographic structure of HA in this composite.

FTIR spectra are shown in Fig. 2. Literature reports that the CHI presents absorption bands at 1657 cm^{-1} (amide I), 1603 cm^{-1} (amine), 1425 cm^{-1} (–COOH), 1319 cm^{-1} (amide III), 1084 cm^{-1},

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