Materials Science and Engineering C 72 (2017) 526–535

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Development, characterization and biocompatibility of chondroitin sulfate/poly(vinyl alcohol)/bovine bone powder porous biocomposite

Gabriela T. da Silva [a], Guilherme T. Voss [b], Vanessa Kaplum [c], Celso V. Nakamura [c], Ethel A. Wilhelm [b], Cristiane Luchese [b], André R. Fajardo [a,*]

[a] Laboratório de Tecnologia e Desenvolvimento de Materiais Poliméricos e Compósitos – LaCoPol. Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas (UFPel), 96010-900 Pelotas, RS, Brazil
[b] Grupo de Pesquisa em Neurobiotecnologia (GPN). Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas (UFPel), 96010-900 Pelotas, RS, Brazil
[c] Laboratório de Microbiologia Aplicada aos Produtos Naturais e Sintéticos and Laboratório de Inovação Tecnológica no Desenvolvimento de Fármacos e Cosméticos, Universidade Estadual de Maringá (UEM), 87020-900 Maringá, Paraná, Brazil

ARTICLE INFO

Article history:
Received 26 July 2016
Received in revised form 14 November 2016
Accepted 21 November 2016
Available online 22 November 2016

Keywords:
Porous materials
Biocomposites
Composites
Chondroitin sulfate
Biomaterial
Tissue engineering

ABSTRACT

Chondroitin sulfate (ChS), a sulfated glycosaminoglycan, poly(vinyl alcohol) (PVA) and bovine bone powder (BBP) were blended to form a novel eco-friendly biocomposite through cyclic freeze-thawing under mild conditions. The systematic investigation reveals that the content of BBP has a remarkable effect on the pore size, porosity, mechanical and liquid uptake properties and biodegradability. At 10 wt.% BBP the biocomposite exhibited enhanced mechanical properties and biodegradability rate as compared to the pristine sample. Further, different properties of the biocomposite can be tailored according to the content of BBP. In vitro assays showed that ChS/PVA-BBP does not exert cytotoxicity against healthy cells. In vivo and ex vivo experiments revealed that ChS/PVA-BBP biocomposites are biocompatibility materials without exert pro-inflammatory responses. The biocomposite was completely biodegraded and bioresorbed after 15 days of treatment. Taken together, BBP is a low-cost source of hydroxyapatite and collagen, which are insurance. All these results suggest that the biocomposite designed in this study is a promising biomaterial for potential skin tissue engineering.

1. Introduction

Innovative and versatile scaffolds had been fabricated in the last years for the most varied purposes (e.g. cell engineering and tissue and bone regeneration) [1,2]. The success of these systems in biotechnological uses is ascribed to the possibility of to mimic a typical in vivo cellular environment [3]. Scaffolds provide nutrients and other factors that allow cells and tissues growing in vitro conditions. Herein, different types of stem cell are seeded in scaffolds to grow and divide as autonomous units. However, in this approach, the cell anchorage on scaffold matrix is required. That means cells will stop growing, dividing and die without anchoring centers. For that reason, among various requirements for an ideal scaffold (adequate architecture, cyto- and tissue-compatibility, bioactivity and good mechanical properties, for instance), the good attachment of anchorage-dependent cells is essential [4]. Scaffolds made of synthetic or natural or both polymers typically provide the necessary structural support for cell attachment and development [1,2]. Plenty of works report on the literature original scaffolds fabricated from several polymers (pure or blended) using different processing techniques [5,6]. In this sense, the glycosaminoglycans (GAGs) are a prominent class of biopolymers used to fabricate scaffolds with controllable chemical, mechanical, and micro structural properties [7].

Chondroitin sulfate (ChS), a sulfated GAG composed of alternating units of (β-1,4)-linked *N*-acetyl-galactosamine (GalNAc) and (β-1,3) glucuronic acid (GlcUA), has captured considerable attention in biotechnological uses due to its numerous biological activities and enhanced physico-chemical properties [8,9]. One of the most important functions of ChS in tissue regeneration is related to its signaling functions of various growth factors and chemokines (i.e. fibroblast and epidermal growth factors) [10]. ChS contributes to stabilization and modulation of the active conformation of these growth factors against fast degradation, for instance. These properties are closely associated with the sulfate groups of ChS [10]. The associations of ChS with other polymers results in reliable scaffolds materials that can absorb growth factors, show good cell adhesion, and avoid undesirable inflammatory reactions at application sites [11,12]. Moreover, ChS allows performing different processing techniques to fabricate scaffolds (i.e. polyelectrolyte complexation, freezing-thawing, chemical and physical crosslinking, etc.) [13,14]. Taking into account these aforementioned aspects, here ChS was blended with poly(vinyl alcohol) (PVA) to fabricate

* Corresponding author.
E-mail address: andre.fajardo@pq.cnpq.br (A.R. Fajardo).

http://dx.doi.org/10.1016/j.msec.2016.11.069
0928-4931/

an eco-friendly hydrogel using the freezing-thawing technique. PVA, a water-soluble synthetic polymer, is commonly used to manufacture biomaterials due to its good mechanical properties, nontoxicity and biocompatibility [15,16]. Thus, ChS/PVA can be considered a potential biomaterial for tissue engineering.

To further enhance the mechanical properties of ChS/PVA we have explored the addition of bovine bone powder (BBP) into the same resulting in a biocomposite material. BBP, a waste material from bovine slaughterhouse, is an eco-friendly and low-cost source of hydroxyapatite and collagen fibrils. Several works make use of collagen or hydroxyapatite in scaffold formulation; in order to endow some required property (i.e. adhesion, biocompatibility, biodegradability, mechanical properties, and so on) [17,18]. To the best of our knowledge a porous biocomposite based on ChS/PVA-BBP has not been explored till date for scaffolding. Here, biocomposites were fabricated using mild conditions and fully characterized in terms of physico-chemical, morphological and water uptake properties; then in vitro tests were carried out to evaluate the cytotoxicity of ChS/PVA-BBP biocomposite against healthy cells. In order to demonstrate the biocompatibility and security of the biocomposites, in vivo and ex vivo experiments were performed.

2. Materials and methods

2.1. Materials

Chondroitin sulfate (ChS) (M_w ~ 20,000 g/mol) was kindly donated by Solabia (Maringá-PR, Brazil). Poly(vinyl alcohol) (PVA) (M_w 84,000–124,000 g/mol, 99% hydrolyzed) and hyaluronidase from bovine testes (type I, 10^3 units/mg) were purchased from Sigma-Aldrich (St. Louis, USA). Slices of adult femoral bovine cortical bones were purchased from the local butcher shop (Pelotas-RS, Brazil). Bovine bone powder was prepared according to the method described by Alves et al. [23]. Briefly, slices of bovine bones were freed from macroscopic fragments manually and by immersion in boiling distilled water. After cooling, they were sonicated in a bath filled with acetone (~15 min). Cleaned bovine bone was oven-dried (100 °C for 24 h) and then, ball milling treated at room temperature for 10 min (Marconi, model MA350, Brazil). The white powder obtained, labeled as BBP, was grounded and sieved through an 80-mesh sieve before use. All other chemicals of analytical grade were used as received without further purification.

2.2. Preparation of ChS/PVA-BBP biocomposites

PVA solution (3.75 wt.%) was prepared by dissolving the polymer in deionized water at 90 °C for 4 h. After the complete solubilization the solution was cooled to 60 °C. ChS solution (8 wt.%) was prepared in deionized water at room temperature for 4 h. PVA and ChS solutions were gently blended and homogenized for 4 h to yield 1:2 PVA/CS weight ratio. Then, different BBP mass contents (0, 1, 5, and 10 wt.%) with respect to ChS/PVA total weight were added in feed solutions, which were stirred for 1 h to homogeneous the system. Afterward, the resulting solutions were cast into plates, which were immediately deep-frozen (−20 °C) overnight and slowly thawed (~5 h). Six freeze-thaw cycles were carried out to physically crosslink the biocomposites, which after the last freeze-thaw cycle were immersed in distilled water overnight prior to eliminate some impurity. After this procedure, the biocomposites (marked here as ChS/PVA-BBP0, ChS/PVA-BBP1, ChS/PVA-BBP5, and ChS/PVA-BBP10, respectively) were recovered and oven-dried at 40 °C for 48 h.

2.3. Characterization techniques

The biocomposites and the neat components (BBP, ChS, and PVA) were characterized by Fourier-Transformed Infra Red (FTIR) spectroscopy, X-ray diffraction (XRD) and Thermogravimetry (TGA) analysis. FTIR spectra were recorded in a Shimadzu spectrometer (model Affinity, Japan) operating in the spectral region of 4000–600 cm^{-1} with resolution of 4 cm^{-1}. The samples were ground with spectroscopic grade KBr and pressed into disks. XRD patterns were measured using a powder diffractometer Siemens (model D500, Germany), with Cu-Kα radiation source (λ = 0.154178 nm) at 30 kV and 20 mA. The scanning range was 5–50° with a scanning rate of 1°/min. TGA curves were obtained using a Shimadzu Analyzer (model DTG60, Japan) with a scanning rate of 10 °C min^{-1} under $N_{2(g)}$ atmosphere with flow of 20 mL min^{-1}in a range of temperature of 25–500 °C. Mechanical properties were investigated by compressive testing using a Texturometer (Stable Micro System, Model TA.TXT2, UK). For this, set of hydrogels samples (cubic shape - edges about 10 mm) were swollen up to equilibrium (~24 h) and then tested in unconfined compressive mode (maximum strain of 95% and crosshead speed of 1 mm/s) at room temperature.

SEM images were recorded using a JEOL scanning electron microscope (model JSM-6610LV, USA) coupled with an energy dispersive X-ray (EDX) analyzer. Prior to the SEM-image record, samples were immersed in PBS (pH 7.4) at room temperature up to the equilibrium swelling (~24 h). Later, these swollen samples (rectangular shape, 1.5 × 0.5 cm) were frozen using liquid nitrogen (5 min of immersion), then, they were removed and carefully fractured (manually) at the middle. The fractured samples were lyophilized in a freeze dryer (Terroni, model LS3000, Brazil) at −55 °C for 24 h. Next, the dried samples were mounted on a stub sample holder and gold-coated by sputtering before SEM/EDX visualization. The microscope was operated at a working distance of around 10 mm and an acceleration voltage of 10–11 kV.

2.4. Porosity

The porosity of each biocomposite was evaluated using a simple liquid displacement method [11]. In brief, a cubic shaped sample was immersed into known initial volume (V_1) of acetone for 24 h at room temperature. After acetone impregnation, the volume of the set hydrogel-ethanol was determined (V_2). Finally, the acetone-impregnated sample was set off and the remaining liquid volume was determined (V_3). The total porosity was calculated using the following equation:

$$Porosity\ (\%) = \frac{(V_1 - V_3)}{(V_2 - V_3)} x\ 100 \quad (1)$$

2.5. Uptake and retention capacities

Here maximum uptake capacity at equilibrium (W_{eq}) was evaluated as function of the percentage of BBP incorporated into the biocomposites formulation. For this, a conventional gravimetric procedure was conducted in each experiment. Briefly, known amounts of each dried hydrogel sample (ca. 100 mg) were pre-weighed and put in a glass vial filled with 100 mL of PBS pH (7.4). Each vial was kept under constant slow stirring at 37 °C for 24 h to reach the swelling equilibrium. The swollen samples were taken from the vials and gently drained to remove the liquid excess. Afterward, those samples were weighed and the W_{eq} parameter was calculated from the following equation:

$$W_{eq}\ (\%) = \left[\frac{w_s - w_o}{w_o}\right] x\ 100 \quad (2)$$

where w_s is the swollen sample weight and w_o is the dried sample weight.

Liquid retention capacity of swollen samples was measured using the follow procedure. Samples were swollen in PBS (pH 7.4) up to equilibrium (~24 h), weighed (w_s) then inserted in a dry centrifuge tube containing filter paper at the bottom. This set was centrifuged at 10,000 rpm for 5 min; the sample was recovered and weighed again