Materials Science & Engineering C 89 (2018) 336–345

Contents lists available at ScienceDirect

Materials Science & Engineering C

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

Silk fibroin/nanohydroxyapatite hydrogels for promoted bioactivity and osteoblastic proliferation and differentiation of human bone marrow stromal cells

Marta Ribeiro[a,b,c,*], Maria H. Fernandes[d,e], Marisa M. Beppu[f], Fernando J. Monteiro[a,b,c], Maria P. Ferraz[g]

[a] i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto 4200-135, Portugal
[b] INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Porto 4150-180, Portugal
[c] FEUP - Faculdade de Engenharia da Universidade do Porto, Departamento de Engenharia Metalúrgica e Materiais, Porto 4200-465, Portugal
[d] Laboratory for Bone Metabolism and Regeneration, Faculdade de Medicina Dentária, Universidade do Porto, Porto 4200-393, Portugal
[e] REQUIMTE/LAQV – U. Porto, Porto, Portugal
[f] School of Chemical Engineering, University of Campinas, Campinas, SP 13083-852, Brazil
[g] FP-ENAS/CEBIMED - University Fernando Pessoa Energy, Environment and Health Research Unit/Biomedical Research Center, Porto 4249-004, Portugal

ARTICLE INFO

Keywords:
Silk fibroin
Nanohydroxyapatite
Bioactivity
Osteoblast differentiation
Bone regeneration

ABSTRACT

Silk fibroin (SF) is a natural, biocompatible, and biodegradable polymer having a great potential for the successful regeneration of damaged bone tissue. In the present work, nanohydroxyapatite (nanoHA) was incorporated into SF polymer to form a bioactive composite hydrogel for applications as bone implants. The degradation and bioactive properties of SF/nanoHA composite hydrogels were evaluated. Additionally, biological investigations of human bone marrow stromal cells (hBMSCs) viability, proliferation and differentiation to the osteoblastic phenotype were conducted. The incorporation of nanoHA in SF polymer matrices improved the bioactivity of the hydrogels. The biological results highlighted that the SF/nanoHA composite hydrogels are suitable for hBMSCs attachment and proliferation, while a test for alkaline phosphatase (ALP) and bone morphogenetic protein 2 (BMP-2) expression suggested osteoblast differentiation. Additionally, a cell staining method for ALP allowed to observe cell infiltration with active production of ALP by the infiltrated cells, paving the way to use the proposed composite hydrogel for bone tissue regeneration.

1. Introduction

Scaffolds and cells are essential components in bone regenerative approaches. These scaffolds focus on developing biologically-based substitutes with similar structure and functionality to the extracellular matrix (ECM) in order to assist cell adhesion and proliferation. Biological scaffolds should gradually degrade to support the cell ingrowth and bone formation through the regeneration process, as well as to avoid the risk of complications that may be associated with the long-term presence of a foreign material [1–4]. Hydrogels have attracted extensive interest because of their advantageous properties similar to those of the native ECM, such as biocompatibility and the ability to absorb high amounts of water or biological fluids without dissolving, thus maintaining their three-dimensional (3D) structure and function. Their high permeability allows the exchange of oxygen, nutrients, and soluble metabolites [5–7].

Silk fibroin (SF) is a protein polymer derived from the cocoons of *Bombyx mori* which possesses adequate properties for bone tissue engineering scaffolds, such as biocompatibility, biodegradability, high permeability to oxygen and water vapor, versatile processing, and adjustable mechanical and biochemical properties [8–11].

SF-based composite hydrogels incorporating relevant molecules of the extracellular matrix such as SF/hyaluronic acid [12] and SF/collagen [13] have been reported to present enhanced physicochemical and biological properties for tissue engineering applications. In a different approach, the incorporation of a bioactive ceramic in the hydrogel matrix is expected to improve the osteogenic potential of the resulting composite. The presence of the bioactive ceramic inside a polymeric matrix would mimic the inorganic phase of the extracellular matrix favoring bone cell behavior and the interaction with the surrounding bone tissue [14,15]. In a previous study, we described the preparation of novel SF-hydrogels incorporating different percentages

* Corresponding author.
E-mail address: ribeiro_marta88@hotmail.com (M. Ribeiro).

https://doi.org/10.1016/j.msec.2018.04.034
Received 18 August 2017; Received in revised form 20 February 2018; Accepted 15 April 2018
Available online 16 April 2018
0928-4931/

of nanophased hydroxyapatite (0, 10, 15, 20 and 30 wt%) by using a new and innovative method, in which ethanol was used as gelling agent [8]. However, when the nanoHA content was higher than 15 wt%, nanoHA aggregation occurred. The SF hydrogel incorporating 15 wt% of nanoHA yielded a composite with improved mechanical properties [8] together with a higher amount of uniformly dispersed particles throughout the matrix, in combination with interconnected micro- and macroporosity suitable for new bone formation. Moreover, in the previous study, non-frozen and frozen hydrogels were evaluated in order to assess differences in the materials properties [8]. The main difference observed was the higher pore sizes for frozen materials (44.4 ± 38.2 μm: min 1.6 μm–max 255.3 μm) compared to non-frozen materials (19.1 ± 18.1 μm: min 1.4 μm–max 104.8 μm). Additionally, preliminary biological data performed with MG63 cells showed promising results regarding osteoblastic cell response [8].

Consequently, the main goal of the current work was to exploit the suitability of the SF/15% wt% nanoHA hydrogel for bone regenerative strategies. For that, the SF/nanoHA hydrogel was prepared as described previously [8] and evaluated for enzymatic degradation, bioactivity and ability to promote the proliferation and osteoblastic differentiation of human bone marrow stromal cells.

2. Materials and methods

2.1. Preparation of silk fibroin solution

Cocoons of *Bombyx mori* silkworm (supplied by Bratac, São Paulo, Brazil) were degummed in 1 g/L Na_2CO_3 solution at 85 °C for 1 h 30 min, with Na_2CO_3 being changed every 30 min to remove the sericin of the cocoons and obtain pure SF fibers. Then, SF fibers were dried and dissolved in a ternary solvent of $CaCl_2$:CH_3CH_2OH:H_2O, in a molar ratio of 1:2:8, at 85 °C until total dissolution, to a SF salt solution of 10% (w/v). The SF salt solution was then dialyzed (cellulose membrane, Viscofan 22 EU – 20 USA) against distilled water for 3 days, at 8 °C, with water being changed every 24 h. The final concentration of the SF aqueous solution was 4% (w/v), which was determined by weighing the remaining solid after drying.

2.2. Preparation of silk fibroin/nanoHA hydrogels

SF/nanoHA hydrogels were prepared according to our previously established method [8]. Briefly, the dry power of nanoHA aggregates (Fluidinova S.A., Maia, Portugal) was first mixed with 70% ethanol and then slowly mixed with the SF aqueous solution at 37 °C. SF and nanoHA were mixed at ratios of SF/nanoHA 100/0 and 85/15 wt%. The hydrogel containing 15% of nanoHA was called SF/nanoHA15. Part of these hydrogels was frozen at −20 °C for 24 h, through one freeze-thaw cycle, to evaluate differences in the properties of non-frozen and frozen hydrogels. The frozen hydrogels were identified with the letter F.

2.3. In vitro enzymatic degradation

In vitro enzymatic degradation of the hydrogels was measured *versus* time, by incubating the gels in protease XIV solution (*Streptomyces griseus*, Sigma) and monitoring the hydrogel mass. Hydrogels sections with 7 mm diameter and 5 mm thickness were carefully transferred to 48-well plates and soaked in phosphate-buffered saline solution (PBS, pH 7.4) overnight to reach swelling equilibrium. The gels were removed from PBS, excess liquid was blotted from the surface with filter paper, and the gel masses were determined. The gels were incubated at 37 °C in 1 mL phosphate-buffered saline solution (PBS, pH 7.4) containing the protease. The enzyme concentrations used in this test were 0.5 and 1.0 mg/mL [16]. The enzyme solution was replaced daily with freshly prepared solution. The control hydrogels were immersed in 1 mL PBS which was also refreshed daily. At designated time points (1, 3, 7 and 10 days), groups of samples were rinsed in distilled water and prepared for mass balance and scanning electron microscopy (SEM).

The percentage of weight loss [W (%)] of hydrogels was determined based on the following equation:

$$W\,(\%) = (W_0 - W_d)/W_0 \times 100$$

where W_0 is the initial weight of the hydrogel sample and W_d is the weight of the sample after degradation at predetermined days.

2.4. Biodegradation and bioactivity assessment in SBF

The *in vitro* degradation and bioactivity of the hydrogels were carried out using standard simulated body fluid (SBF) containing inorganic ion concentrations similar to those of human blood plasma [17]. The SBF solution was prepared by dissolving NaCl (8.035 g), $NaHCO_3$ (0.355 g), KCl (0.225 g), $K_2HPO_4.3H_2O$ (0.231 g), $MgCl_2.6H_2O$ (0.311 g), $CaCl_2$ (0.292 g) and Na_2SO_4 (0.072 g) into 700 mL ultrapure water. The solution was buffered at physiological pH 7.4 with Tris buffer (6.118 g) and HCl. Then the total volume of solution was filled up to 1000 mL with ultrapure water [17].

Hydrogels sections with 7 mm diameter and 5 mm thickness were carefully transferred to 48-well plates and were soaked in ultrapure water overnight to reach swelling equilibrium. The gels were removed from ultrapure water, excess liquid was blotted from the surface with filter paper, and the gel masses were determined. Then, the samples were immersed separately in 20 mL SBF in closed falcon tubes at 37 °C for 1, 3, 7, 14 and 21 days. After the different incubation time-points, the materials were removed from SBF solution, washed with ultrapure water and prepared for mass balance. Finally the hydrogels were freeze-dried, sectioned and viewed using SEM for apatite layer formation.

2.5. SEM

SEM was used to observe the morphology of samples after enzymatic degradation with protease XIV solution, as well as the apatite layer formation in the hydrogels after immersion in SBF. The analysis was performed on samples sputter coated (SPI-Module) with a thin gold/palladium film and then examined by SEM using a FEI Quanta 400 FEG/ESEM (FEI) scanning electron microscope at an accelerating voltage of 15 kV.

2.6. In vitro biological studies

2.6.1. hBMSCs isolation and culture

Human Bone Marrow Stromal Cells (hBMSCs) were obtained from bone marrow following orthopaedic surgery procedures, with patient's informed consent (male patients, 25–45 years old). The bone fragments (which would be otherwise discharged) were broken in small pieces and washed with alpha minimum essential medium (α-MEM, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 IU/mL/2.5 μg/mL penicillin-streptomycin solution (Gibco) and 2.5 μg/mL amphotericin B (Gibco). The cell suspension was seeded in Petri dishes for 10 days. Afterwards, at 70–80% confluence, the cell monolayer was washed with PBS twice and cells were detached with trypsin solution (0.04%, Gibco) and subcultured. All assays were conducted with cells in passage 4.

Prior to cell seeding, the hydrogel sections with 7 mm diameter and 5 mm thickness were sterilized in ethanol solution at 70% (v/v) and subsequently washed twice with phosphate-buffered saline (PBS). For cell seeding, a suspension of 10^5 cells/scaffold was added on the top of each hydrogel. For the osteogenic medium, the above described medium was supplemented with 10 mM β-glycerophosphate (Sigma) and 10^{-8} M dexamethasone (Sigma). The seeded scaffolds were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. All samples were cultured for 1, 7, 14 and 21 days. The hBMSCs-seeded hydrogels were assessed for cell viability, proliferation, alkaline phosphatase (ALP) activity, total protein content, and F-actin cytoskeleton,