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Development of silk fibroin/nanohydroxyapatite composite hydrogels for bone tissue engineering

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ABSTRACT

This work presents a novel composite hydrogel consisting of silk fibroin (SF) and nanohydroxyapatite (nanoHA) prepared by a new and innovative method using ethanol as gelling agent capable of forming hydrogels in few minutes. The properties of the composite material, such as the microstructure as well as the chemical and physical properties were studied. Moreover *in vitro* studies of osteoblastic citocompatibility were performed. The microporosity and macroporosity obtained combined with interconnected porous structure and a uniform dispersion of nanoHA particles throughout the fibroin matrix makes composite hydrogel suitable for bone regeneration. The compression modulus of composite hydrogels was increased as the nanoHA concentration increased from 10 to 15 wt.% and the water uptake ability of these materials decreased with the incorporation of nanoHA. The metabolic and alkaline phosphatase activities of osteoblastic cells were improved with the incorporation of nanoHA in the SF matrix providing a more promising material for bone tissue engineering.

1. Introduction

Bone tissue engineering has emerged as a promising alternative in cases of bone loss, overcoming problems of rejection and donor scarcity associated to the clinical used bone grafts [1,2]. By combining three-dimensional structures (3D) – scaffolds, cells and growth factors, bone tissue engineering seeks to achieve a long lasting and fully functional regeneration of bone [3]. Materials with high hydrophilic properties appear suitable for mimicking the aqueous *in vivo* environment. For this reason, hydrogels have been used extensively as 3D matrices [4]. They represent promising systems for the healing and regeneration of damaged tissues since they are highly permeable and facilitate the transport of nutrients and metabolites [4]. Their ability to mimic body tissues and respond to external stimuli has made them important and promising forms of biomaterials for various applications including tissue engineering, controlled drug release devices, etc. [5]. Current research on biodegradable polymers is emerging, combining these structures with osteogenic cells, as an alternative to autologous bone grafts. Different types of biodegradable materials have been proposed to be used as 3D porous scaffolds for bone tissue engineering. Among them, natural polymers are one of the most attractive options, mainly due to their similarities with extracellular matrix (ECM),

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chemical versatility, good biological performance and inherent cellular interactions [6]. The unique properties of silk fibroin (SF), a protein polymer isolated from the cocoons of the domestic silkworm *Bombyx mori*, such as slow biodegradation, adjustable mechanical properties, low inflammatory response, high permeability to oxygen and water vapor, resistance to enzymatic degradation, favorable processability in combination with biocompatibility, have driven wide interest in this material for a variety of applications, ranging from textiles to biomedical use [7–10]. Based on these features, interest has arisen in the use of *B. mori* SF as starting material for biomaterials and scaffolds for bone tissue engineering. The cocoon of the silkworm is mainly composed of sericin and fibroin. Sericin is a glue-like protein that holds SF fibers together in the cocoon case. SF is composed of a repetitive sequence of amino acids: glycine, alanine and serine, and is not soluble in water due to its high concentration of hydrophobic amino acids [7]. SF has two types of molecular conformation of the secondary structure, called silk I and silk II. Silk I is a metastable form of SF that is soluble in water and non-crystalline; random coil and α-helix conformations are usually called silk I. On the other hand, silk II is a highly stable and organized structure that is insoluble in water; the β-sheet conformation is called silk II. Generally, both silk I and silk II are present in SF products, but it is their relative proportions that will define the final properties [11–13]. Due to the β-sheet formation, SF exhibits relatively slow degradation *in vitro* and *in vivo* when compared to collagens and many other biopolymers [7,14]. The biodegradability, mechanical integrity and low inflammatory response of SF [15] ensure its role as one of the promising porous materials for osteogenic applications. In addition, studies demonstrate that SF scaffolds are able to induce calcium phosphate deposition in *in vitro* calcification experiments, demonstrating that SF is a promising scaffold for bone regeneration [16,17]. We hypothesize that the incorporation of nanosized HA particles (nanoHA) into biodegradable SF hydrogels should improve osteogenic outcomes. Hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] is one of the most widely used synthetic calcium phosphate ceramics due to its chemical similarities to the inorganic component of hard tissues and it possesses exceptional biocompatibility, bioactivity and osteoconductivity [18–22]. Some studies have indicated that nanostructured materials may promote increased specific protein interactions to more efficiently stimulate new bone growth compared to conventional materials [23,24]. NanoHA shows increased potential to bind to bone, to adsorb macromolecules that may act in the preliminary events leading to bone bonding and tissue regeneration [25,26]. The aim of this work was to develop a novel composite hydrogel for bone tissue engineering of silk fibroin and nanoHA, an approach that has been poorly explored. The hydrogels were prepared by a new and innovative method, which can be used to form hydrogels in few minutes, using ethanol as gelling agent. The focus of the present study was to accelerate the formation of hydrogels without occurrence of nanoHA aggregation or sedimentation. The attributes of SF in combination with the features of nanoHA will form a material with interesting properties, combining the mechanical integrity and slow degradation of SF with the bioactivity and osteoconductivity of the nanoHA.

2. Materials and methods

2.1. Preparation of silk fibroin solution

Cocoons of *B. mori* silkworm were supplied by Bratac (São Paulo, Brazil). The cocoons were degummed three times by soaking in 1 g/L of Na_2CO_3 solution at 85 °C for 30 min to remove the sericin of the cocoons, and then rinsing in distilled water to remove Na_2CO_3 residues. The 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 changes every 24 h [27]. 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 composite hydrogels

For preparing the SF/nanoHA hydrogels, a total of 3.5 mL of SF aqueous solution was placed in specific molds (diameter: 25 mm). The dry powder of nanoHA aggregates (Fluidinova S.A., Maia, Portugal) was first mixed with 1.5 mL of 70% ethanol and then slowly mixed with the SF aqueous solution using a pipet to avoid protein precipitation. The weight percent (wt.%) of nanoHA in the hydrogels was 0, 10, 15, 20 and 30. The hydrogels were named according to their nanoHA content. SF/nanoHA hydrogels were prepared at two temperatures. The molds were sealed and kept at a controlled temperature in a thermostatic bath at 37 or 50 °C until hydrogel formation. Gelation time was determined when the sample showed an opaque white color and did not flow when the mold was inverted for 30 s [28,29]. Part of these hydrogels was frozen at −20 °C for 24 h to evaluate differences in the properties of non-frozen and frozen hydrogels. These hydrogels were identified with the letter F. Moreover, since fibroin is a natural polymer, the gelation time was evaluated from SF solutions prepared in different days to evaluate the reproducibility of the materials and to find a highly reproducible method for the formation of hydrogels. The data reported for gelation time represent the average of ten replicates for each temperature.

2.3. Characterization

2.3.1. Morphology

Morphology of non-frozen and frozen hydrogels was characterized by scanning electron microscopy (SEM). The analysis was performed on samples that were frozen with liquid nitrogen, lyophilized, coated with a gold layer and then examined with an EVO MA15 scanning electron microscope (Zeiss, England), with an accelerating voltage of 10 kV. The average pore size of the materials was