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Composite of porous starch-silk fibroin nanofiber-calcium phosphate for bone regeneration

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Abstract

New bioactive nanobiocomposite scaffolds based on silk fibroin nanofiber-porous starch are presented for potential bone tissue regeneration. The silk fibroin nanofibers were fabricated directly via wet electrospinning using methanol coagulation bath and then the chopped electrospun nanofibers were incorporated into the starch matrix, followed by particulate leaching and freeze-drying. To achieve bioactivity, the calcium phosphate was then deposited throughout the fabricated scaffolds via alternate soaking in saturated calcium and phosphate solutions at 37 °C. The morphology, structure, swelling, and calcium phosphate forming ability of the scaffolds were evaluated and the results indicated that addition of silk fibroin nanofibers into the starch matrix reduced the mean pore size, porosity, and water uptake of the fabricated scaffolds. Moreover, the deposited calcium phosphate layer consists of both brushite and apatitic calcium phosphate. The highest amount of formed calcium phosphate is evident in the starch matrix and increasing the amount of silk fibroin nanofibers decreases calcium phosphate formation. Cell culture experiments with osteoblast-like cells (MG63) on the scaffolds coated with calcium phosphate demonstrated that incorporation of SF nanofibers into the starch hydrogel improves cell viability, proliferation, and attachment.

Keywords: Porous starch; Silk fibroin; Wet electrospinning; Calcium phosphate deposition; Bone regeneration

1. Introduction

Despite the progresses achieved in various fields of orthopedics, large bone defect therapy has remained a challenge. Over the past few decades bone tissue engineering (BTE) has been developed in order to regenerate and repair damaged sites with biological substitutes [1]. Beside the need for osteogenesis cells and signaling agents, scaffolds play an important role in formation of neo-bone in BTE. The scaffold should consist of biocompatible and biodegradable materials with interconnected pores to increase oxygen permeability and nutrient supply to deeper areas. Moreover, osteoconductive properties of scaffolds facilitate osteoblast cells spread, growth and new bone generation [2]. Various synthetic and natural porous polymeric materials have been used extensively for bone tissue regeneration. Natural biodegradable polymers including polysaccharides and proteins are often used due to their cytocompatibility and similarity to extracellular matrix (ECM) of native tissues [3].

Among different kinds of natural polymers, starch is the most promising material in the field of biomedical engineering, since it is biocompatible, cost-effective, and widely available. However, the major disadvantages of the starch-based materials using starch taken alone for BTE purposes are their lack of processability and high water sensitivity [4]. In this regard, in order to enhance starch matrix performance in biomedical engineering and biotechnology fields, there is a growing trend towards incorporating natural polymeric fibers into the starch matrix, including bacterial cellulose [5], plant cellulose [6], and bamboo [7]. In addition to these approaches, fiber-reinforced composites have gained much popularity in recent years for BTE scaffolding due to its similarity to bone structure [8]. Nano-scale fibers have been suggested to be effective reinforcing agents because of their resemblance to the fibrous structures of bone tissue ECM and large surface area, which enhance cell attachment, proliferation, and growth [9]. In the past few years, electrospinning has been

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considered as an effective method for producing various polymeric nanofibers [10]. Thus, designing porous starch-based composite by incorporating electrospun biopolymer nanofibers appears to be a promising approach to improve its properties to mimic the bone ECM structure.

Silk fibroin (SF) is a kind of fibrous biopolymer with slow degradation rate, which shows remarkable biological and mechanical properties in both in vitro and in vivo conditions. It has been proven that the slow degradation rate of SF materials along with the high oxygen permeability make them suitable for slow regenerating of growing tissue such as bone [11]. Regarding these features, various forms of SF such as nanofibers are widely studied for bone tissue scaffolding [12]. Based on our knowledge, no previous study tested effects of SF nanofibers addition on the starch-based hydrogels.

The further requirement for starch-based BTE scaffolds is the ability to bond with surrounding tissue. However, homogenous distribution of ceramic fillers in polymer matrix is challenging and weak binding between ceramics and polymer have been reported. Coating of polymeric surface with various calcium phosphates (CaPs), especially apatite, is a common approach in order to achieve bioactive BTE scaffolds [13]. Taguchi et al. [14] proposed that alternate soaking process is a simple and feasible method for homogenous coating of CaPs in three-dimentional porous polymeric structure due to its ability to control the thickness of formed apatite layer through changing reaction cycles.

This study aims to develop and study a set of new bioactive composite scaffolds from silk nanofiber-starch hydrogel for bone regeneration applications. Accordingly, the SF nanofibers were first prepared by wet electrospinning using methanol bath and then added to starch matrix by using glutaraldehyde as a crosslinker. Afterwards, CaP was deposited throughout the fabricated porous composites via alternate soaking in saturated calcium and phosphate solutions at 37 °C. Various techniques such as scanning electron microscopy (SEM), X-ray diffraction (XRD), and IR spectroscopy were used to analyze the structure and morphology of fabricated composites and the deposited CaP. Besides structural characterization, the biological response of MG63 osteoblast-like cells cultivated on the surface of starch-based scaffolds coated with CaP was evaluated.

2. Experimental

2.1. Materials

Silk cocoons from *Bombyx mori* were generously provided by the Iranian silkworm research center. Besides, lithium bromide (LiBr; 746479, Sigma, Saint Louis, USA), sodium carbonate (Na_2CO_3; 106392, Merck, Germany), methanol (822283, Merck, Germany), cellulose dialysis tube (12 KDa, MWCO, Sigma, Saint Louis, USA), glycine (104169, Merck, Germany), glutaraldehyde 25% (820603, Merck, Germany), isopropanol (818766, Merck, Germany), hydrochloric acid fuming 37% (HCl; 100317, Merck, Germany), absolute ethanol 99.7% (Hamoon Teb Markazi, Zarandieh, Iran), formic acid (100264, Merck, Germany), and potato starch (Golha, Iran) were purchased.

Table 1
The code and composition of each fabricated composite scaffolds.

SF nanofibers content (%)	Scaffold samples	
	Before CaP deposition	After CaP deposition
0	ST	ST-CP
5	ST-5SF	ST-5SF-CP
10	ST-10SF	ST-10SF-CP
15	ST-15SF	ST-15SF-CP

2.2. Preparation and characterization of SF electrospun nanofiber

SF was extracted from *B. mori* cocoons based on previous reports [15]. Briefly, *B. mori* cocoons were boiled in 0.02 M aqueous Na_2CO_3 solution for 1 h to remove sericin, washed with deionized water and dried at 37 °C overnight. The degummed silk was dissolved in 9.3 M LiBr at 60 °C for 4 h, dialyzed in a cellulose tube against distilled water for 72 h and then lyophilized (MC4L, UNICRYO, Freeze-dryer, Germany). The prepared SF sponge was then dissolved in formic acid under stirring for 3 h at room temperature to yield 13 wt% SF solution. Subsequently, the obtained solution was filled in 1 ml syringe with 22 G blunted stainless steel needle. Electrospinning (Fanavaran Nano-Meghyas, Iran) was carried out at 20 kV with a constant flow rate of 0.25 ml h^{-1}. The electrospun fibers were collected in the methanol coagulation bath for direct recovery of β-sheets. The methanol bath was placed 13 cm under the needle tip. To determine morphology and size, the fiber mat was attached on an aluminum stub, sputter coated with gold, and imaged with a scanning electron microscope (SEM; Essen Philips XL 30). SEM images from approximately 50 random fibers were analyzed to determine the average fiber diameter and their distribution using the image analysis software (ImageJ; National Institutes of Health, Bethesda, Maryland, USA). Fourier transform infrared spectroscopy in the attenuated total reflectance mode (FTIR-ATR, Equimo55 bruker FTIR spectrometer) was used to examine the SF structural changes during electrospinning in methanol coagulation bath. The quantitative analysis of amide I absorption band within the 1600–1700 cm^{-1} region [16,17], was done using curve-fitting and de-convolution methods to determine the amount of α-helix, β-sheet, and random coil elements in each spectrum.

2.3. Preparation and characterization of SF nanofiber-starch composite scaffolds

The three-dimentional (3D) porous nano-biocomposites from pure starch and SF nanofibers were prepared via particulate leaching and freeze drying methods. Potato starch (12 g) and different amounts of chopped electrospun SF nanofibers were added to 100 ml distilled water containing 75 µl ml^{-1} glutaraldehyde and stirred thoroughly for 20 min. Starch and SF nanofibers were mixed with different weight ratios of 100/0, 95/5, 90/10, and 85/15. Afterwards, the pH of the solution was adjusted to 2 by