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Preparation and characterization of silk fibroin/collagen sponge with nanohydroxyapatite

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ABSTRACT

A freeze drying technique was used to form a porous three-dimensional silk fibroin/collagen/hydroxyapatite sponge. Structure of sponge was analyzed using IR spectroscopy. Mechanical properties of sponge were tested and compared with composites containing different ratios of components in samples. Microstructure of porous 3D scaffolds was analyzed by Scanning Electron Microscope. Results showed that scaffolds made of blend of silk fibroin and collagen with inorganic particles of nanohydroxyapatite were less flexible and more stiff than biopolymeric sponge without inorganic part. Moreover, microstructure of those samples was less regular and the size of pores was bigger.

KEYWORDS

Silk fibroin; collagen; hydroxyapatite; tissue engineering

Introduction

Biomaterials used in tissue engineering for bone regeneration should mimic the structure and properties of natural bone and should contain both organic and inorganic parts. Bone tissue is composed mainly of type I collagen and nanohydroxyapatite crystals [HAp, $Ca_{10}(PO_4)_6(OH)_2$] [1]. Additionally, bone contains other organic part such as proteins, polysaccharides and lipids [2]. In living organism bone has very important property such as self-healing. Unfortunately, some defects resulting from trauma or those caused by tumor resection need an orthopedic support [3]. For such support several biomaterials can be used for example: hydrogels [4], thin films [5], microspheres [6] or scaffolds [7].

To obtain matrices for tissue regeneration many different polymers can be used. For this purpose both synthetic polymers and natural ones have been already employed [8]. Natural polymers have advantages like non-toxicity, biocompability, biodegrability [9]. Collagen is a natural polymer, it is a protein which naturally occurs in human body mainly in extracellular matrix of bones and tendons tissue [10]. In literature 29 types of collagen have been reported so far, but the most abundant in human organisms is collagen type I. Therefore, collagen type I is widely used in various applications, mostly in cosmetics, medical, food and pharmaceuticals products [11]. Sometimes blends of two polymers may be more interesting as a material because they may show other more suitable properties for medical applications [12].

Silk is built by two major proteins: silk fibroin (fibrous protein) and sericine (globular protein). For biomedical application sericine must be removed from silk to eliminate the

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immunology response of human body [13]. After the degummed process, silk fibroin is highly biocompatible protein. Some of the medical devices made with silk fibroin have been approved by Food and Drug Administration [14].

Hydroxyapatite is an inorganic component in bone tissue. It is widely used in tissue engineering as an additive for implants [15]. Collagen and silk fibroins are proteins and their miscibility were confirmed by us using viscometric analysis [16]. Silk fibroin/collagen/ hydroxyapatite composites were already prepared [17,18]. A novel porous silk fibroin/collagen/hydroxyapatite scaffold with interconnected pores was fabricated by NaCl particulate leaching method by Mou et al. [17]. Chen et al. used $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$ to obtain hydroxyapatite crystals in silk fibroin/collagen hydrogels [18].

The aim of this work was preparation of 3D silk fibroin/collagen scaffold modified with different amount of nanohydroxyapatite. The properties of scaffold important in biomedical applications were studied.

Materials and methods

Preparation of silk fibroin/collagen solutions

Silk fibroin (SF) was obtained from *Bombyx mori* cocoons in our laboratory following the method described by Kim et al. with slight modifications [19]. Empty cocoons were boiled two times in 0.5% Na_2CO_3 for 1 hour. After the removing of solution, silk fibroin was washed for 5 min in deionized water and boiled in 5% alkaline soap solutions and 20 min in deionized water. These steps were repeated for 3 times. 4% solution of silk fibroin were prepared by dissolving polymer in $CaCl_2$:H_2O:C_2H_5OH (molar ratio 1:8:2) at 80°C for 4 h according to procedure found in the literature [20]. Collagen (Coll) was obtained in our laboratory from rat tail tendons. Tendons were washed in deionized water and dissolved in 0.1M acetic acid for 3 days in 4°C. Undissolved parts were centrifuged for 10 min at 10,000 rpm [16, 21]. The obtained solution was frozen at −18°C and lyophilized at −55°C and 5 Pa for 48 h (ALPHA 1–2 LD plus, CHRIST, Germany). Collagen was then dissolved in 0.1M acetic acid to obtain 1% wt. solution.

Fabrication of silk fibroin/collagen/hydroxyapatite sponge

Silk fibroin and collagen solutions were mixed together in weight ratio: 100:0, 90:10, 75:25. All mixtures were dialyzed (SERVAPOR dialysis tubing MWCO 1200-1400) against deionized water for 3 days with changing deionized water 2 times per day. After dialysis process, 20 and 50% nanohydroxyapatite (Sigma-Aldrich) was added to the mixture to obtain composite. Three-component mixture was placed in a plastic container and frozen at -80°C to avoid sedimentation of suspension. 3D scaffolds were obtain during the lyophilisation process for 2 days. The properties of samples were then analyzed.

ATR-FTIR spectroscopy

The structure of scaffolds was evaluated by attenuated total reflection infrared spectroscopy using Nicolet iS10 equipped with an ATR device with diamond crystal. All spectra were recorded in absorption mode at 4 cm^{-1} intervals and 64 scans.

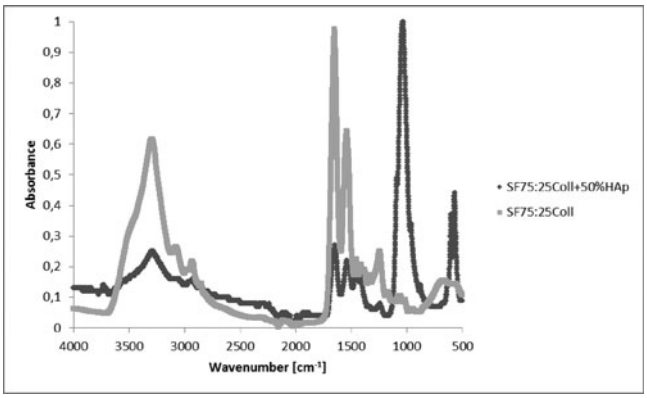

Figure 1. FT-IR spectra for SF/Coll sponge with and without hydroxyapatite.

Mechanical properties

Mechanical properties of samples were measured by using Zwick&Roell 0.5 testing machine with crosshead speed set at 0.5 mm/min. 5 samples of each kind were placed between two disc and pressed. Compression strength and Young modulus were evaluated. Mechanical properties of all samples were studied in dry condition.

Scaffold morphology

The size of pores and their distribution were analyzed based on Scanning Electron Microscope (SEM) pictures. Samples were prepared by freezing each kind of scaffold in liquid nitrogen for 3 min and then they were cut with a razor scalpel. Based on SEM pictures, the distribution of crystals was analyzed.

Results and discussion

IR spectra for silk fibroin/collagen scaffold with and without hydroxyapatite addition are shown in Fig. 1. Silk fibroin and collagen are proteins, so their characteristic bands in IR spectra are similar. Silk fibroin is built of two polypeptide chains linked by a disulfide bridge. In composition of heavy chain of silk fibroin the most abundant amino acid is glycine and the most repeated sequence is Gly-Ala/Ser dipeptides [22]. Collagen molecule is built of three chains, with a repeating primary sequence of (Gly-X-Y)n where as X and Y the most often proline and hydroxyproline are present [23, 24]. For proteins typical bands in IR specta are N-H stretching at 3305cm^{-1} (Amide A), C-H stretching at 3081 cm^{-1} (Amide B), C=O stretching at 1635 cm^{-1} (Amide I), N-H deformation at 1545 cm^{1} (Amide II). In IR spectra of all kind of samples obtained in our study those characteristic bands were observed. After the addition of hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$) to biopolymer samples, the characteristic band related to phosphate can be observed. It is located between 900 cm^{-1} and 1200 cm^{-1}. In SF/Coll (75:25) +50% HAp the bands from phosphoric groups are located at 1086 cm^{-1}, 1032 cm^{-1}, 961 cm^{-1}. Moreover, peaks at 599 cm^{-1} and 563 cm^{-1} also represent phosphoric groups. Roveri et al. [15] suggested that using IR spectra the interaction between collagen and hydroxyapatite can be assessed. After addition of HAp a peak corresponding to –COO^{-} changed its position to lower wavenumber, because new bond between Ca^{2+} from HAp and –COO^{-} from collagen is formed [15].

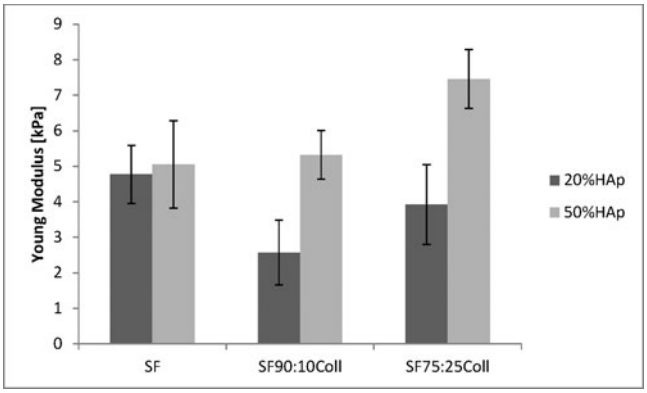

Figure 2. Young modulus of silk fibroin/collagen/hydroxyapatite scaffolds in different ratios.

Mechanical properties of scaffolds based on the blend of silk fibroin and collagen with HAp were tested in dry condition in room temperature. Young modulus and compressive strength were measured and compared (Figs. 2–3). Scaffolds with hydroxyapatite addition showed smaller flexibility in comparison to sponge made of proteins only. It is especially clearly showed after 50% HAp addition to biopolymer mixture. The value of Young modulus is smaller for SF/Coll with 20% HAp. For samples with addition of 50% HAp bigger value of Young modulus in comparison to the samples with 20% HAp in composite was observed. With increasing amount of HAp in composite the flexibility of sponge decreases. Young modulus increases also with addition of collagen to silk fibroin/HAp composite. Sponge made of SF has smaller Young modulus (about twice) compared to sponge made of SF with hydroxyapatite. Young modulus for SF sponge is similar when it contains 50% HAp and 20% HAp: 5.05 kPa and 4.77 kPa, respectively. For SF/Coll+20%HAp sponges Young modulus is smaller in comparison to SF-HAp sponge. For sponge SF90:10Coll+50% HAp Young modulus is 5.32 kPa and is two times bigger in comparison to SF90:10Coll+20% HAp (2.57 kPa). The value of Young modulus for scaffold made of SF75:25Coll with 50% hydroxyapatite addition is 7.46 kPa, and it is almost two times bigger than the value of Young modulus for sample with 20% addition of HAp.

Compressive strength of biopolymeric scaffolds is similar for both 20% and 50% addition of inorganic particles to biopolymer blend. For samples with 20% HAp the addition of collagen causes the decrease of compressive strength almost twice comparing with SF+20% HAp.

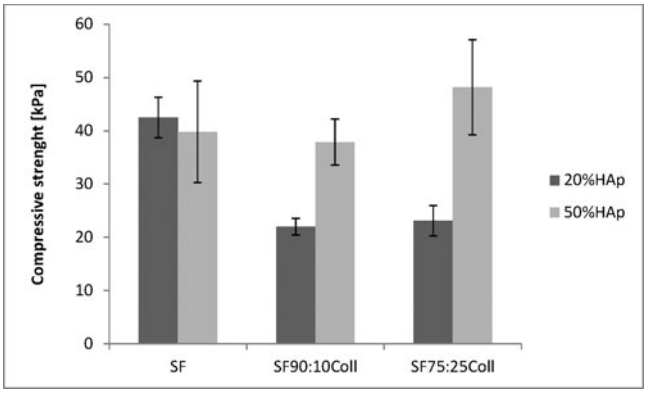

Figure 3. Compressive strength of silk fibroin/ collagen/ hydroxyapatite scaffolds in different ratios.

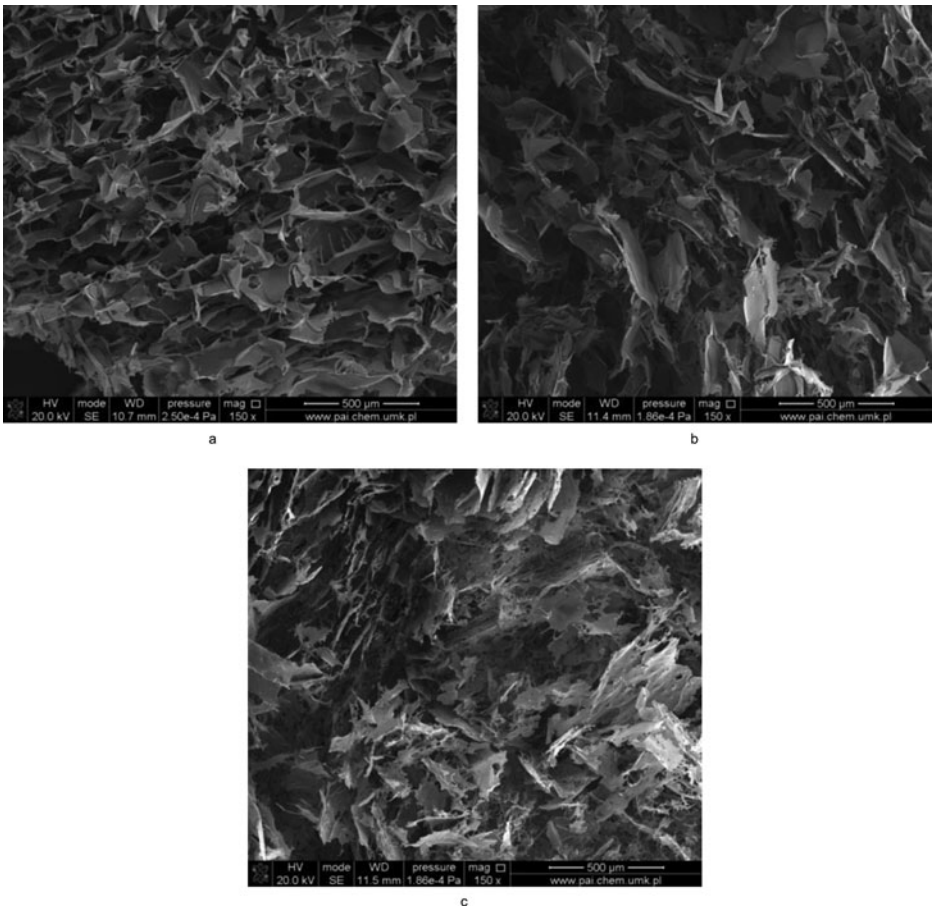

Figure 4. Scanning Electron Microscope picture of scaffolds with 20% HAp addition in 150 magnification: a) SF, b) SF90:10Coll, c) SF75:25Coll.

The biggest value of compressive strength among all studied specimens with inorganic parts was observed for SF75:25Coll+50% HAp (48.2 kPa). It is over two times bigger in comparison to the specimen with 20% addition of hydroxyapatite (23.08 kPa). For SF sample the compressive strength is bigger in comparison to SF-HAp samples. Sample made with pure SF is more resistant for crush. The decrease of compressive strength after inorganic particles addition can be caused by a small volume of proteins that can interact with those crystals. 3D specimens with bigger addition of hydroxyapatite are less flexible and more stiff. After crushing they loss their shape, especially those with 50% HAp.

The microstructure of scaffolds based on blends of silk fibroin, collagen and HAp was observed using Scanning Electron Microscope. The pictures were obtained under 150 times magnifications (Fig. 4). As one can see in the pictures all 3D specimens prepared in this study possess porous structure. Sample of SF+20% HAp has the most regular microstructure among all studied specimens (Fig. 4a). Moreover, the size of pores and distribution of pores are similar to microstructure of pure SF scaffold (showed in our previous study) [16]. After addition 50% of hydroxyapatite to sample, pores became larger and more irregular (Fig. 5). Additionally, micropores inside the scaffold can be noticed. With increasing amount of collagen in sponge the pores become more irregular. When 50% addition of hydroxyapatite is in the scaffold then the specimen shows more "sheet- like" structure. The pores distribution may also

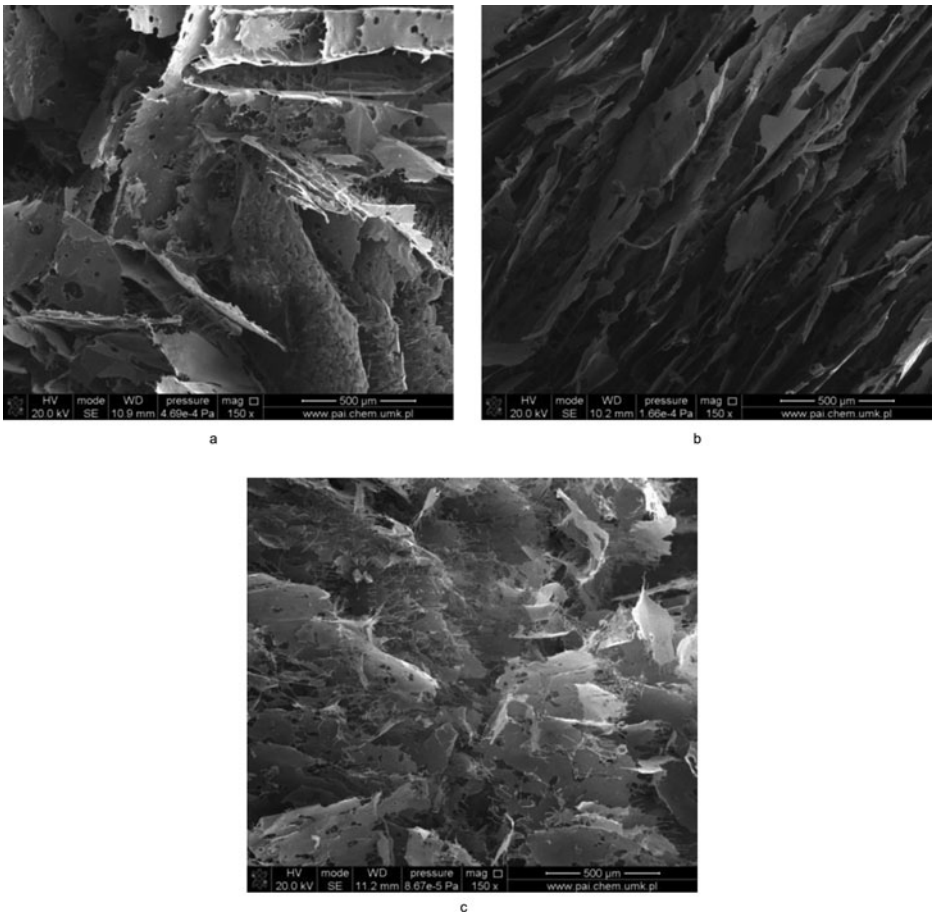

Figure 5. Scanning Electron Microscope picture of scaffolds with 50% HAp addition in 150 magnification: a) SF, b) SF90:10Coll, c) SF75:25Coll.

affect the mechanical properties of sponges. Sheet-like structure can be one of the reason of low compression strength of 3D samples [16].

Conclusions

3D scaffolds based on blends of silk fibroin/collagen and nanohydroxyapatite can be prepared using freeze drying technique. The presence of hydroxyapatite in scaffolds was confirmed by IR spectroscopy. With increasing ratio of inorganic part in silk fibroin/collagen samples, the increase of Young modulus was observed. Silk fibroin/collagen sponges became less flexible and more stiff when contain hydroxyapatite. Moreover, after addition of nanohydroxyapatite to silk fibroin/collagen sponge, the structure of pores is less regular. The scaffold can be potentially used in medical and cosmetic field, however, biological study need to be completed.

Acknowledgments

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