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Preparation and characterization of silk fibroin/collagen sponge modified by chemical cross-linking

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ABSTRACT

3D scaffolds made of blend of silk fibroin and collagen were obtained by lyophilisation process and cross-linking with EDC/NHS. The structure of the scaffolds was studied by ATR-FTIR spectroscopy. Morphological properties were analyzed by SEM. Porosity for cross-linked samples were measured. The mechanical properties of samples were tested in dry and wet conditions and compared with non-modified samples.

The results showed that the blend of silk fibroin and collagen after cross-linking with EDC/NHS and lyophilisation has porous structure. Mechanical properties were improved by cross-linking with EDC/NHS. Moreover, the cross-linked scaffolds have got better stability in water than non cross-linked ones.

KEYWORDS

Silk fibroin; collagen; crosslinking; EDC/NHS

Introduction

One of the main problems of human health are accidents and illnesses that cause a loss of organ or tissue [1]. Tissue engineering tries to resolved this problems by using natural and synthetic polymeric matrices to produce appropriate shape of biomaterial. There are many of structures that can be used as biomaterials e.g. thin films, gels, 3D sponges, micro- and nanoparticles [2]. Collagen is a biopolymer widely used in medical field due to its unique properties. Collagen can stimulate the migration and infiltration of cells and support their proliferation [3]. Moreover, collagen is the major protein in extracellular matrix (ECM) and composes ca 30% of protein in human body [4,5]. Unfortunately, non-modified collagen degrades quickly in *in vitro* condition [6] and provides non suitable mechanical properties to be applied as biomaterials [7]. Silk proteins are produced by variety species of silkworms, spiders, scorpions and bees [8]. Silk fibers are built by two proteins, namely, fibroin and sericine. Sericine is the gummy substance surrounding fibroin [9]. For biomedical applications only silk fibroin is useful, sericin due to its immunogenicity needs to be removed [1]. Collagen and silk fibroin are miscible and material based on the blends of these two polymers was already made [10-14]. To provide better properties of such material the cross-linking agent can be used. For cross-linking of proteins one can use physical and/or chemical process. As physical cross-linking

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agent UV irradiation, gamma radiation or temperature may be applied [15, 16]. As chemical cross-linking agent many substances were used so far, e.g. genipin [17, 18], polyethylene glycol diglycidylether (PEG-DE) [19], glutaraldehyde acetals [20] or EDC/NHS [21-22]. 1-Ethyl-3-(3-dimethyl-aminopropyl-1-carbodiimide) (EDC) is often used with N-hydroxysuccinimide (NHS). Mixture of these solutions causes the formation of amide bond and therefore the cross-linking of proteins can be achieved. Moreover, EDC is not incorporated into a chain and by-product of these reaction, urea, can be easily removed from the matrices by rinsing with water [23]. The aim of this work, was to prepare 3D sponges based on silk fibroin and collagen and modification of their properties by EDC/NHS as crosslinking agent.

Materials and methods

Preparation of silk fibroin/collagen mixtures

Silk fibroin (SF) was obtained from *Bombyx mori* cocoons in our laboratory following the method described by Kim et al. with slight modifications [24]. 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. Collagen (Coll) was obtained 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 [10, 25]. 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 dissolved in 0.1M acetic acid to obtain 1% wt solution. Silk fibroin and collagen were mixed together in 90:10, 75:25, 50:50 SF/Coll weight ratio. Pure silk fibroin was left as control sample. 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 the mixture of polymers was placed in polystyrene container and frozen. 3D scaffolds were obtain during the lyophilisation process for 2 days.

Scaffold crosslinking

Porous sponges were placed in methanol for 4h at room temperature to induce crystallization and to improve water stability of samples [1,12,26]. Samples were left overnight to methanol evaporation. 5% solution of cross-linking agent was prepared in 98% ethyl alcohol. Samples were cross-linked in EDC/NHS solution for 2h. After the removal of cross-linking agent, scaffolds were immersed in sodium phosphate dibasic 0.1 M Na_2HPO_4 two times for 30 min. Sodium phosphate dibasic was changed then with deionized water. Samples were placed in deionized water for 2h with changing water every 20 min. After these processes, scaffolds were frozen and lyophilized. Dry samples were analyzed and compared with samples without cross-linking.

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.

Table 1. The position of characteristic bands in IR spectra of silk fibroin/collagen sponge (SF/Coll)before and after cross-linking with EDC/NHS.

	Observed vibrational frequencies wavenumber [cm^{-1}]							
Assignments	SF	SF (EDC/NHS)	SF:Coll 90:10	SF:Coll 90:10 (EDC/NHS)	SF:Coll 75:25	SF:Coll 75:25 (EDC/NHS)	SF:Coll 50:50	SF:Coll 50:50 (EDC/NHS)
H_2O adsorbed, N—H (amide A)	3283	3279	3277	3279	3294	3279	3283	3279
C—H (amide B)	3078	3077	3067	3077	3079	3077	3078	3077
H_2O adsorbed, C=O (amide I)	1645	1622	1641	1622	1650	1622	1648	1622
N—H (amide II), C—N	1537	1515	1516	1515	1540	1515	1536	1515
C—N, N—H (amide III)	1241	1231	1236	1231	1242	1231	1238	1231

Table 2. Porosity (Є) and density (d) of scaffolds.

Sample	W [mg]	d [mg/cm^3]	Є [%]
SF	58,77 ± 0,93	75,95 ± 3,58	76,69 ± 11,17
SF90:10Coll	53,83 ± 0,25	115,52 ± 8,82	84,64 ± 12,02
SF75:25Coll	48,4 ± 1,15	83,66 ± 2,51	65,97 ± 3,04
SF50:50Coll	32,06 ± 0,21	45,93 ± 3,91	42,67 ± 6,15

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. Compressive strength and Young modulus were evaluated. Mechanical properties of all samples were studied in dry condition. Samples without cross-linking dissolved in PBS. Therefore, mechanical properties of cross-linked samples were checked in dry and wet conditions. Scaffolds were placed in a chamber with PBS buffer (pH = 7.4) and mechanical properties in wet state were tested.

Porosity and density

Porosity and density were studied for all cross-linked samples. These parameters were analyzed using liquid displacement method with ethanol as a liquid [27]. Porous sample with a known weight (W) was immersed in a cylinder with a known volume of ethanol (V_1) for 5 min. The volume of ethanol with sample was measured (V_2) and the volume of liquid after the removal of scaffold (V_3) was measured too. Each kind of samples were measured in triplicate. The density (d) and porosity (Є) of scaffolds were obtained using the following equation:

$$\epsilon = (V_1 - V_3) / (V_2 - V_3)$$
$$d = W / (V_2 - V_3)$$

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.

Results and discussion

ATR-FTIR spectroscopy

Both, silk fibroin and collagen are proteins and for this reason in IR spectra these biopolymers show similar bands. Therefore, it is difficult to assess the miscibility of those polymers by using FTIR spectroscopy. Miscibility of silk fibroin and collagen were confirmed earlier by us usingviscometric measurements [10]. For proteins, amide bands are characteristic such as Amide I, II, III and Amide A and B. Silk fibroin in sponges showed typical random coil conformations and α-chains with peaks at 1645, 1537 and 1241 cm^{-1} which corresponds to Amide I, II and III bonds. Depending on conformations of protein the characteristic bands in IR spectra may differ. For α-chelix Amide I and II occur at 1655 and 1537cm^{-1}, respectively. Silk fibroin

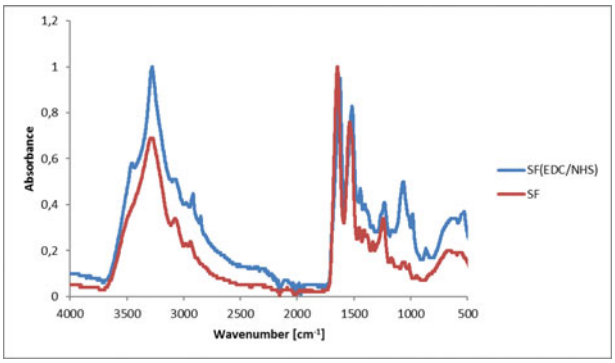


Figure 1. ATR-FTIR spectra of SF and SF modified with EDC/NHS.

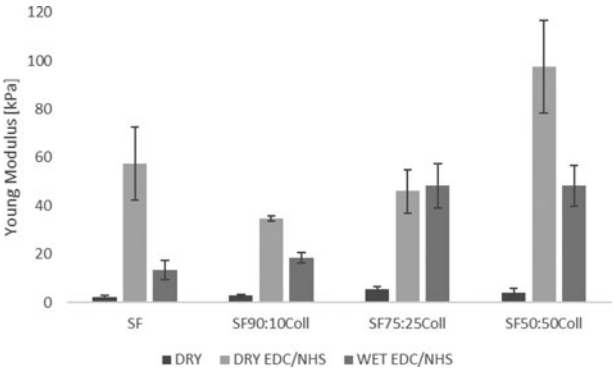


Figure 2. Young Modulus of SF/Coll sponge treated and non-treated with cross-linking agent in wet and dry conditions.

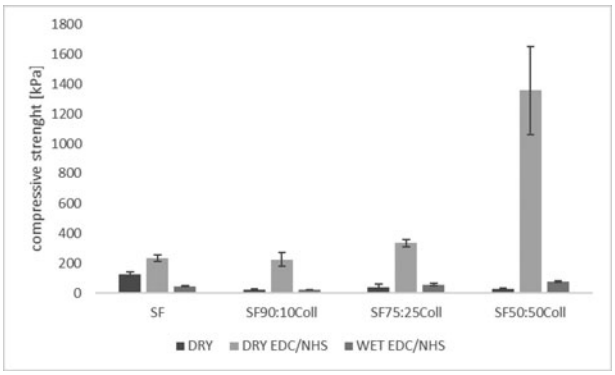


Figure 3. Compressive strength of SF/Coll sponge treated and non-treated with cross-linking agent in wet and dry conditions.

sponges cross-linked by EDN/NHS were soaked first in methanol. Methanol induced β-sheet conformation of silk fibroin therefore sharp peaks at 1625cm^{-1} for Amide I and at 1515cm^{-1} for Amide II are observed [28]. Moreover, characteristic peaks in cross-linked silk fibroin sponges are more intense than for non-cross-linked SF sample (Fig. 1). Scaffolds made of silk fibroin and collagen cross-linked with EDC/NHS show the same position of characteristic peaks in IR spectra (Table 1). There is no shifts between them. Differences can be seen only

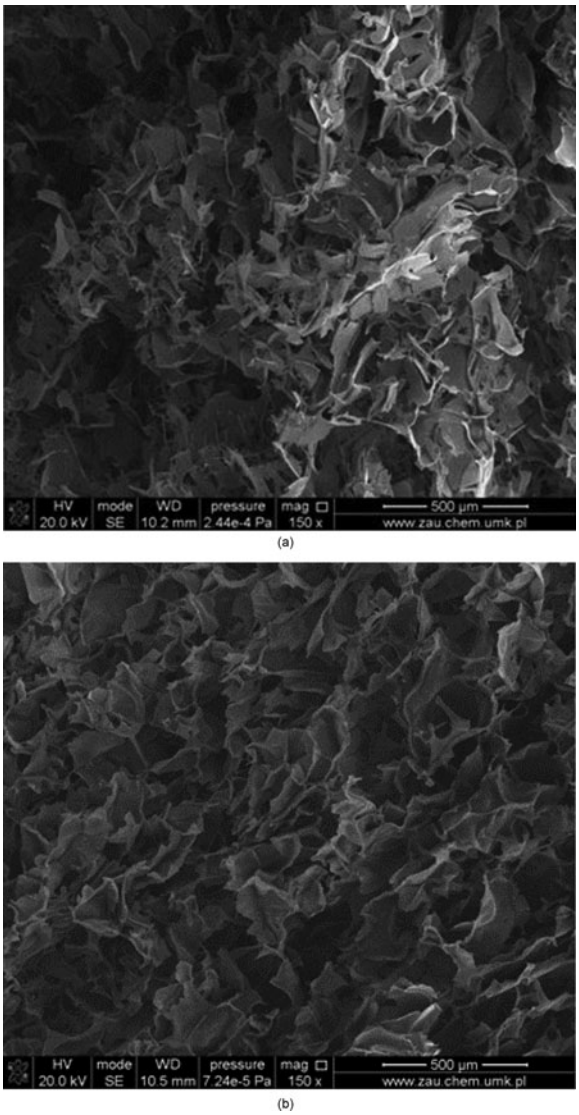
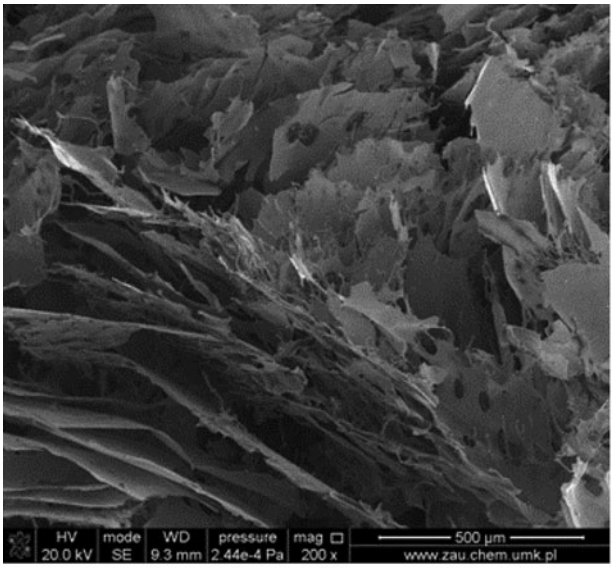


Figure 4. Scanning Electron Microscope pictures of SF samples under 150 magnification: a)Non-cross-linked, b) Cross-linked with EDC/NHS.

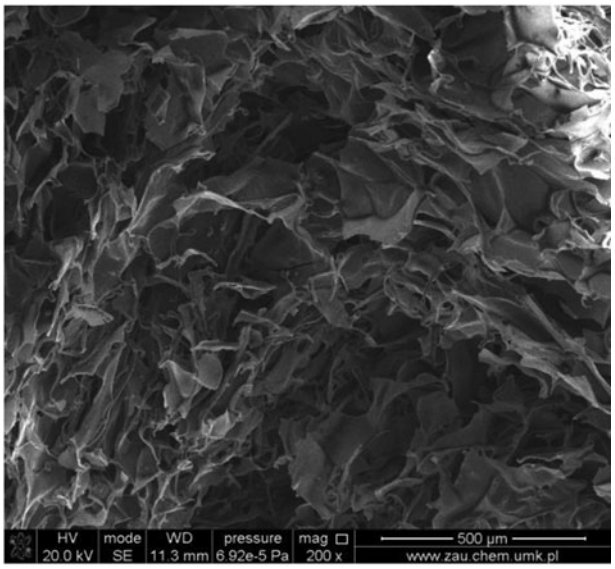
between non-cross-linked and cross-linked samples based on the blends of silk fibroin and collagen.

Mechanical properties

Mechanical properties of obtained materials in dry and wet conditions were tested. Both, Young's Modulus and compressive strength were measured. In wet condition mechanical properties were studied only for cross-linked scaffolds, as for non-crosslinked ones the dissolution process in PBS was observed. Results of Young Modulus are shown in Fig. 2. When we compare the values of Young Modulus for samples in dry conditions, the difference up 10 to 25 times can be noticed. Non-modified samples are elastic and after compressing they return to their original shape. Cross-linked samples are more rigid and less flexible. Both



(a)



(b)

Figure 5. Scanning Electron Microscope pictures of SF90:10Coll samples under 200 magnification: a)Non-cross-linked, b) Cross-linked with EDC/NHS.

kind of samples (cross-linked and non-cross-linked ones) show that Young Modulus value depends on collagen content in samples. The value of this parameter increases with increasing amount of collagen in the specimen. In dry state Young Modulus value of SF50:50Coll is ca. three and two times bigger than for SF90:10Coll and SF75:25Coll, respectively. Analyzing cross-linked samples in wet and dry conditions the values of Young Modulus is bigger for dry samples than for wet ones. Results for compressive strength are shown in Fig. 3. Analyzing compressive strength for modified and non-modified samples in dry conditions one can see that for each sample after EDC/NHS treatment the value of compressive strength is bigger than before cross-linking. After cross-linking the compressive strength for SF sponge

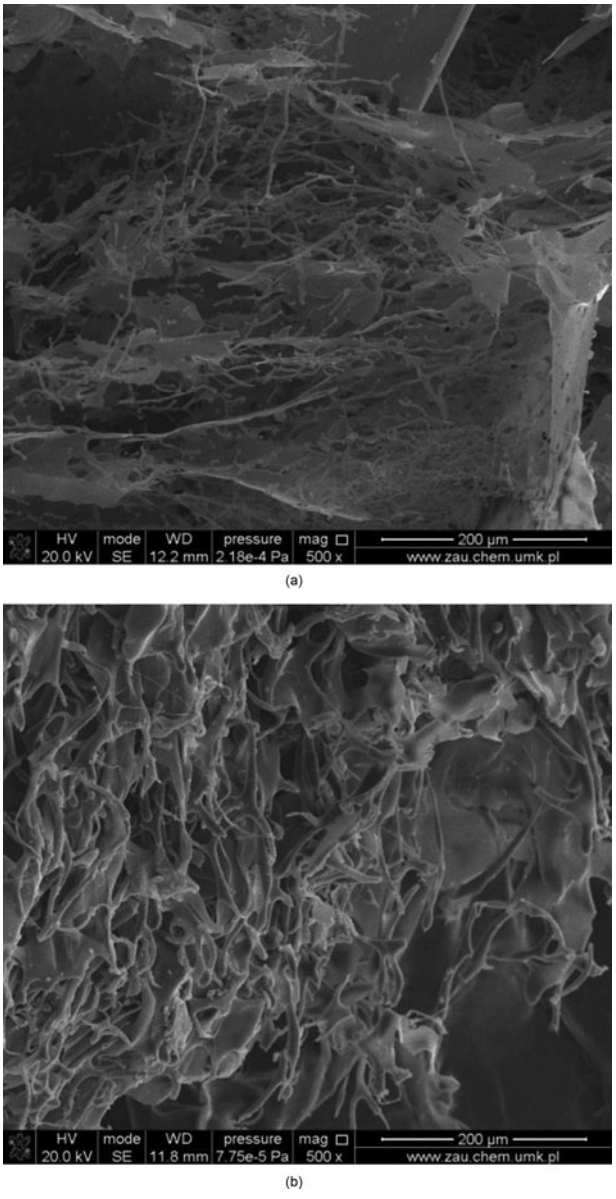


Figure 6. Scanning Electron Microscope pictures of SF75:25Coll samples under 500 magnification: a) Non-cross-linked, b) Cross-linked withEDC/NHS.

increased almost twice from 124 kPa to 234 kPa. Differences in compressive strength values for samples with collagen addition are even bigger. Compressive strength of SF90:10Coll and SF75:25Coll increases ca. 10 times. For SF50:50Coll the value of this parameter increased 50 times. The value of compressive strength for dry samples treated with EDC/NHS for SF50:50Coll is 1356 kPa and is over 5 times bigger when compare with SF, SF90:10Coll and SF75:25Coll modified with EDC/NHS. Mechanical properties were also tested in wet condition in chamber filled with PBS buffer. After soaking in PBS buffer samples swell and become more flexible. The value of compressive modulus decreases and samples are less stiff. Cross-linked sponges in wet state have smaller compressive strength of about 5 to 17 times. Modified

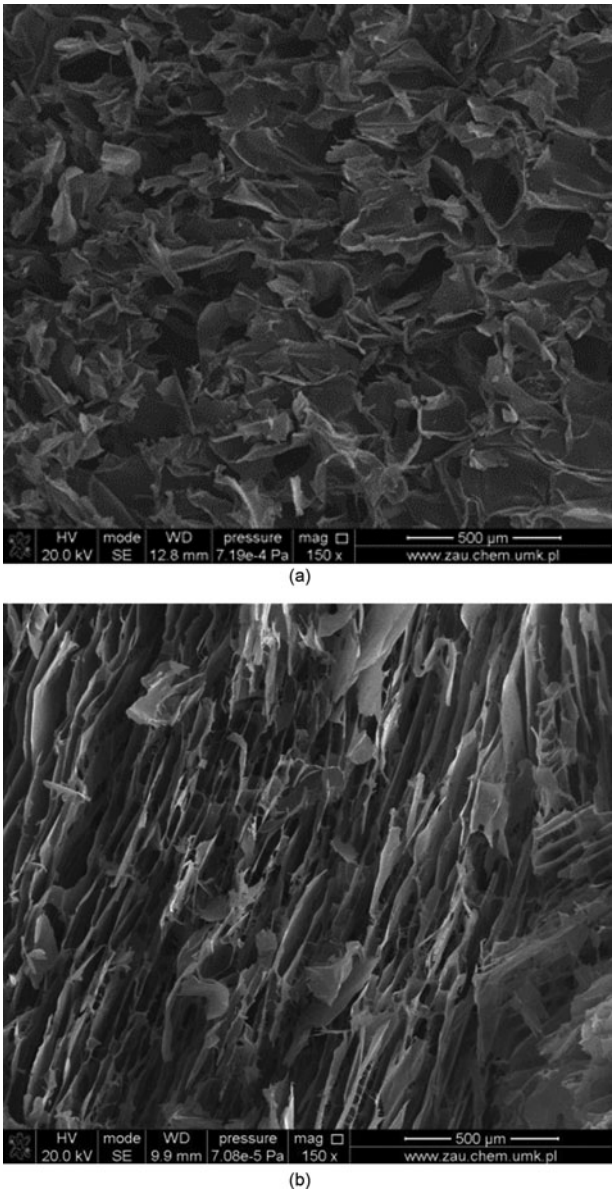


Figure 7. Scanning Electron Microscope pictures of SF50:50Coll samples under 150 magnification: a) Non-cross-linked, b) Cross-linked with EDC/NHS.

samples SF50:50Coll under both wet and dry conditions show bigger values of compressive strength in comparison to samples with smaller collagen content in the blend.

Porosity and density

Porosity and pore size are very important for samples which are considered to be applied as a bone substitute. Scaffolds should be made with porous material to provide good environment for cell growth and proliferation. Porosity and density were analyzed for cross-linked samples by liquid displacement method. The highest value of porosity 84% was found for sample with small additive of collagen (Table 2). With increasing content of collagen in sample the

decrease of porosity was observed. The smallest value of porosity was found for sponge made of SF50:50Coll. Scaffolds for bone tissue regeneration should have sufficient porosity, ca. 90% to allow osteoblast and osteoprogenitor cells to growth [21]. For scaffolds made of SF and SF90:10Coll, the porosity was 76% and 84%, respectively. It may suggest that those scaffolds due to high porosity can be applied in bone tissue regeneration.

Scaffold morphology

Scaffold morphology was analyzed based on Scanning Electron Microscope pictures (Fig. 4–7). The microstructure of modified scaffold is more organized comparing with non-cross-linked sponges. Samples without EDC/NHS modifications show sheet-like structure and pores are long and thin which may cause a low value of compressive modulus [10]. After the cross-linking of samples with EDC/NHS they show more ordered structure. The arrangement of pores does not look like sheets anymore, therefore, mechanical properties of cross-linked samples are much better.

Conclusion

3D porous scaffold made of the blend of silk fibroin and collagen were modified with 1-Ethyl-3-(3-dimethyl-aminopropyl-1-carbodiimide/N-hydroxysuccinimide (EDC/NHS) as cross-linking agent. After cross-linking process of scaffolds the pore arrangement was altered and mechanical properties of materials were improved. Moreover, the cross-linked scaffolds have got better stability in water than non cross-linked ones. The porosity of scaffolds can be sufficient for bone tissue engineering. The biocompatibility of scaffolds should be tested in biological study.

Acknowledgments

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