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DESIGNING AND CONSTRUCTION OF SILK, POLYVINYL ALCOHOL, GELATIN NANO-SCAFFOLD FOR USING IN ARTICULAR CARTILAGE TISSUE ENGINEERING

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ABSTRACT

The cartilage is a type of connective tissue, supporting the soft tissues due to strength of its extracellular matrix, which allows this tissue tolerate the mechanical pressures without permanent deformation. Due to its smooth elastic surface, it gives slippery and impact property. In this research, combined silk fibroin-gelatin -polyvinyl alcohol scaffolds were developed and studied for cartilage tissue engineering. Silicon fibroin silk and gelatin are used as a bed for cell adhesion and proliferation. In this research, the nanofibrous silk fibroin-gelatin- poly-vinyl alcohol scaffolds were prepared using electrospinning method. First, silk fibroin-gelatin solution with a ratio of 70 to 30% and a separate 8% poly-vinyl alcohol solution were prepared, and then, nano-fibers were constructed through two nozzles by using electrospinning method. Then, imaging was performed using scanning electron microscopy, infrared spectroscopy, biodegradation tests and tensile strength test. After qualitative confirmation of nano-fibers, cell culture of cartilage cells was performed on the scaffold for 30 days. Then, imaging by scanning electron microscopy, hematoxylin-eosin staining, and measurement of glycosylation of amino glycan were performed. The silk fibroin / gelatin / poly-vinyl alcohol scaffold supports the cell growth and chondrogenic phenotypes, indicating a relative expression of type II against collagen Type I. Cartilage cells have grown on the scaffold and synthesized glycosaminoglycan. The results of examining the scaffold revealed that the mean diameter of the fiber was 155.75 nm and the biodegradability was 7% after three weeks. In the examinations related to FTIR silica fibroin, we had three peaks of functional groups related to silk fibroin, so the presence of silk fibroin in the silk fibroin scaffold is confirmed. In examinations related to FTIR gelatin, we have three peaks related to the gelatin functional group, so the presence of gelatin in the silk fibroin scaffold is confirmed, and the presence of the OH group confirms the presence of poly vinyl alcohol. In the studying the cell growth by hematoxin-eosin staining, it is shown that at the end of the third week, 20 cells (counting at 200 micrometers) have been grown. The results indicate that silk fibroin-gelatin-poly vinyl alcohol scaffolds might be an appropriate alternative to artificial cell scaffolds for cartilage tissue engineering.

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Introduction

Cartilage is a semi-solid connective tissue, stemming from embryonic mesenchyme. It has elastic structure, found mainly at the end of bones where the joints are located, and it functions as facilitating of joint movement [1,2]. Scaffolds were used for cell culture by Naton et al in 1990. It involved the extracellular culture of cells on polymer-poly-glycolic acid. Accordingly, cells of skin, liver, pancreas, bone marrow, osteoblasts and chondrocytes were used for three-dimensional culture in the system. The Naton suggestion was proposed here, so that these cultured cells along with the scaffold to be transplanted to the living environment or the patient's body. Naton et al (1992) conducted study on the hip joint. Accordingly, they used it as a cover of cartilage cells in three-dimensional form. Then, they replaced the prosthesis in the patient's body. Then, they work on the scaffolds and construction of artificial tissues, such as skin bone marrow, respiratory tract, pancreas, and bladder, were accelerated rapidly, and National Science Foundation was leading in this regard [3,4].

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When a tissue is injured, the injured tissue might be restored using its own stem cells. However, if the severity of the injury is so much that it leads to loss of stem cells, the injured tissue can be restored using the laboratory facilities and using tissue engineering. Tissue engineering is a method, in which the cells are taken from a patient and they are seeded on a scaffold after culture and increasing their number. Proper chemical, biological, mechanical, and electrical stimulation is applied and new tissue is formed within short time. Theoretically, we would able to construct any tissue using this method [5]. As cartilage is a non-vein tissue, it has limited self-restoration capability, and chondrocytes are spherical in Invivo, but they are in fibroblast and stretched in Invitro and they lose their round shape and gene expression might be altered. Therefore, an appropriate and ideal scaffold maintaining the round phenotype of chondrocytes seems to be an essential.
Thus, in addition to selecting an appropriate cellular source for cartilage tissue engineering, designing an engineered cartilage element, for example, a type of scaffold on which cells are placed, is necessary. Physical elements such as design, micro pores, micro pores, and topography of an engineered element play an important role in changing its biological function. Hence, an appropriate scaffold in cartilage tissue engineering, as with other tissues, needs to include a set of key elements, which are biodegradable and environmentally compatible. Moreover, the pore needs to be mechanically stable and allow the cell pass through and guide the extracellular signals [6, 7-11].

Different methods for construction of scaffold

Scientists have used various techniques for repetitive construction of scaffolds in various tissue engineering applications since the mid-80s. The scaffold construction technique is determined based on final properties of the mass and material surface and the expected performance of the scaffold. In addition, the time and cost used for construction of scaffold are considered as vital factors. In scaffold construction techniques, heat or pressure applying and the polymer dissolution in the solvents are used for preparation of the polymer. In this regard, the conditions should be provided so that the bioactive molecules bioactive, used in construction of the scaffolds, is not damaged .The use of bioactive molecules of scaffold and its topical release of drugs and gene from it is considered another important consideration in the preparation of scaffolds. Bioactive molecules such as proteins, peptides similar to extracellular matrix, and DNA can contribute in cell cohesion and signaling and release of the gene and drug, and the technique used to prepare the scaffold should not damage these molecules. The use of drugs and genes is also considered as an appropriate method to improve the time and quality of the restored tissue by improving the migration, cohesion, growth and differentiation of cells. Each of the techniques used for construction of scaffolds has its disadvantages and advantages. The selection of method to construct the scaffold depends on requirements and properties of the target tissue.

Methodology

Cocoons were purchased first. Then, healthy cocoons were removed from the defective cocoons and the silkworm was removed and the cocoons were washed with much water. We placed them in the oven to be dried. Then, the cocoons were crushed. In order to isolate the silicone in the silk, silk was degummed. Accordingly, the cocoons derived from the previous stage were added into boiling water and sodium bicarbonate was poured to it and it was boiled for half an hour. The cocoons were dehydrated and re-boiled with the above method in $NaHCO_3$. The cocoons were washed three times with distilled water. Then, samples were dehydrated and transferred to the oven to be dried [1-18].

Fibroin dissolution

We dissolve the silk degummed in the previous step in a solvent of three ethanol / water / calcium chloride solvents with ratios of 1:8:2. The solution obtained from the fibroin dissolution stage was poured into treated dialysis bag and placed it in a beaker containing distilled on a stirrer with low speed. We change the distilled water of the beaker every 3 to 4 hours. After 3 days, we empty the fluid in the bag and lyophilize the resulting fluid from the fluid [14, 17].

Silk fibroin fiber – gelatin solution preparation

First, the ratio of 70 to 30 with a concentration of 13 w / w of silk gelatin-fibroin is prepared. For the preparation of 4 milliliters of silk fibroin-gelatin, 0.36 g of silk freeze-dried in 3.48 ml of trifluoric acid (TFA) solvent is dissolved. Then, 0.15 g of gelatin is added to it, and remains in room temperature for three hours to obtain a homogeneous solution [11,16].

Silk fibroin solution electrospinning

A syringe containing a mixed solution of silk-fibroin-gelatin with ratio of 9:4 was placed on a syringe pump and the exit speed was set on 0.5 ml / h. The nozzle distance to the collector was considered to be 160 cm. The voltage of 19 kv was applied to the two heads of electrode connected to the nozzle and the collector. Accordingly, the solution underwent electrospinning and the nano-fiber mesh was collected on the collector plate [11, 16].

Silk fibroin-gelatin – Poly vinyl alcohol solution preparation

Experiment treatments:

1-Preparation of silk fibroin – gelatin solution:

First, the ratio of 70 to 30 with a concentration of 13 w / w of silk gelatin-fibroin is prepared. For the preparation of 4 milliliters of silk fibroin-gelatin, 0.36 g of silk fibroin freeze-dried in 3.48 ml of trifluoric acid (TFA) solvent is dissolved. Then, 0.16 g of gelatin is added to it, and remains in room temperature for three hours to obtain a homogeneous solution [9].

2-Poly vinyl alcohol preparation:

First, 0.8 g of poly vinyl alcohol is dissolved in 100 ml distilled water. Then, the obtained solution is placed under heat for 24 hours on the stirrer so that the vinyl alcohol to be solved homogeneously [11, 9].

Silk fibroin-gelatin- poly vinyl alcohol electrospinning

A syringe containing a mixed solution of silk-fibroin-gelatin with ratio of 9:4 was placed on a syringe pump and the exit speed was set on 0.5 ml / h. The nozzle distance to the collector was considered to be 160 cm. The voltage of 19 kv was applied to the two heads of electrode connected to the nozzle and the collector. Another syringe containing poly vinyl alcohol solution is placed on the syringe pump and the exit speed is set on apparatus. The distance of nozzle to collector is considered 100 cm. Accordingly, the solutions underwent electrospinning and the nano-fiber mesh was collected on the collector plate [11,16]. The voltage of 8 kv was applied to the two heads of electrode connected to the nozzle and the collector [8]. To examine and observe the structure and size of the constructed scaffold fibers, SEM is used. In addition, to observe the chemical groups on the constructed scaffold structure, FTIR is used. In addition, to examine the level of tolerating the tension applied to scaffolds, mechanical tension test apparatus was used. To examine the degradation rate of the scaffold at one-week time intervals, biodegradability test is used.

Cell culture

Cartilage samples were taken from the calf joint and transferred to the laboratory and washed with PBS. Then, trypsin enzyme was removed and samples were placed in collagenase enzyme for 16 hours. After 16 hours, the cells were transferred to the flask containing the culture medium and the flasks were placed inside the incubator (0.5: CO_2 and 37 ° C). The cell culture medium should be replaced once per three days [15]. The culture medium included:

DMEM (Dulbecco s modified Eagles) 45 ml:

FBS (Fetal Bovine serum) 4.5 ml:

AA (Antibiotic-Antimycotic (penicillin-streptomycin) 45 µl:

Treatment of scaffolds:

The constructed scaffolds should be treated before that cultured cartilage cells to be transferred on them. For this reason, scaffolds are placed in ethanol 70% and ammoniac 7%.

Transfer of cartilage cells on scaffold:

Scaffolds are punched first. Then, the scaffolds are sterilized. Ethanol is used to sterilize the scaffolds. Then, the cultures' medium is removed and the trypsin enzyme is poured on the, to separate the cells. Then, about 30,000 cells are counted for each scaffold. The punched scaffolds are placed inside the 48-cell plate.

Then, cells are added to it. Finally, they are paced inside the incubator. The cell culture medium is replaced every three days and the new medium is inoculated to them. Once per week, one of the scaffolds is removed and left for examination [10, 15]. SEM is used to examine the cells on the scaffolds.

DMEM(Dulbecco s modified Eagles): 40 ml

FBS(Fetal Bovine serum): 4.5 ml

AA(Antibiotic-Antimycotic(penicillin-streptomycin) 45 µl:

Proline: 0.5 µl

Sodium pyruvate: 0.5 µl

Dexamethasone: 100 µl

Cell examinations:

Hematoxylin-eosin staining method:

As most of the stains used are water-soluble and act only in aqueous environments, the tissues cut of paraffin blocks can be directly applied to aqueous solutions and stained. Thus, it is necessary to paraffinize these cuts before staining and replace the paraffin within it with water in order to absorb the stains soluble in the water. This action is performed during steps in contrast to what is performed in dehydration and clearing the tissue.

Glycosaminoglycan measurement method

The sample is first crushed. Then, about 200 µl of papain enzyme (25 mg/ml of papain, 2 µg/ml of cysteine, 50 µg/ml of NaH_2 Po4, 2 µg/ml of EDTA at pH of 6.5) is poured on it so that samples to be digested. Then, samples are placed at 65 ° C for 24 hours. Then, 400 µ, 0.1 M sodium is poured on the obtained samples and kept at room temperature for 30 minutes.

The extract is neutralized by adding 400 µl HCl 0.1 M, 100 µg/ml. First, the standard sample absorption rate (calf thymus) needs to be measured by micro plate reader at 530 nm at different concentrations and its chart is plotted. Then, the concentration rate of glycosaminoglycan in the samples is measured by a micro plate reader at 530 nm [13].

Results

Results of scanning electron microscopy of synthesized scaffolds:

As shown in Figure (A), fibroin-silk-gelatin nano fiber has been formed in a form of interconnected mesh with a non-grained uniform morphology. The mean diameter fibroin silk-gelatin nano fiber is about 57.9 nm.

In an investigation reported by Gui-Bo Yin et al, they could construct silk fibroin-gelatin polymers and cultured fibroblast cells on the scaffold. Given the obtained results, the ratio of 30: 70 and the concentration of 11% and the solubility of formic acid were accepted and the cells had been grown well on the constructed scaffold.
Nano-fiber resulting from a three-component silk-gelatin- poly vinyl alcohol has a non-grain uniform morphology with an average diameter of 155 nm. As seen, in the two-component mesh, compared to three-component mesh, the smaller average diameter has been obtained. Figure B.

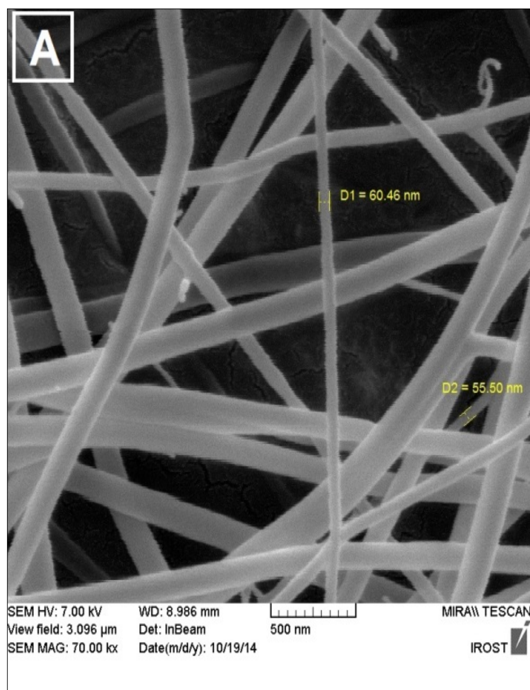

Figure A. silk fibroin-gelatin

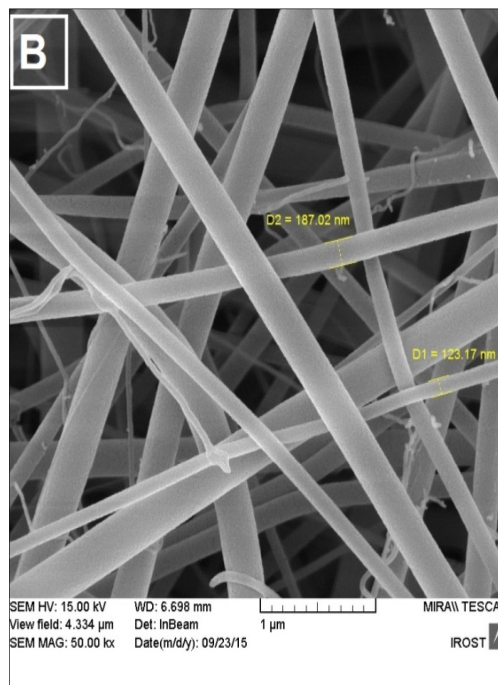

Figure B. Silk fibroin-gelatin- poly-vinyl alcohol scaffold

FTIR results of synthesized scaffolds

In investigations related to FTIR, silk fibroin has three functional groups of amide 1, amide 2, and amide 3, in which the wave number of the functional group of amide 1 is in the range of 1623-1656, the wave number of the functional group of amide group 2 is in the range of 1538 -1530 and the amide (3). As shown in Chart A, we also have three peaks stated in these ranges, indicating the presence of functional groups in the silk fibroin. Thus, it confirms the presence of silk fibroin in silk fibroin scaffold.
In investigations related to gelatin FTIR, gelatin has three functional groups (OH), (N-H) and (C-H), which the wave number of the functional group (OH) is in 3289.97, the wave number of the functional group (N-H group) is in 3073.46, and the wave number of the functional group is in 2936.50. As seen in the chart, we also have three peaks reported in the chart, indicating the existence of functional groups in the gelatin. Thus, it confirms the presence of gelatin in the silk fibroin scaffold.
In investigations related to FTIR, silk fibroin has three functional groups of amide 1, amide 2, and amide 3, which the wave number of the amide group 1 is in the range of 1623-1656, the wave number of the amide group 2 is in the range 1538-1530 and amide 3. As shown in chart A, we also have three peaks, indicating the existence of functional groups in the silk fibroin. Thus, it confirms the presence of silk fibroin in the silk fibroin scaffold.
In investigations related to gelatin FTIR, the gelatin has three functional groups (OH), (N-H) and (C-H), which wave number of OH (OH) is in 3289.97, the wave number of the functional group (N-H) is in 3073.46 and the wave number of the functional group of(C-H) is in 2936.50.
As seen Chart B, we also have three peak stated in the chart, indicating the presence of functional groups in the gelatin. Thus, it confirms the presence of gelatin in the silk fibroin scaffold and the presence of OH functional group confirms the presence of poly vinyl alcohol.

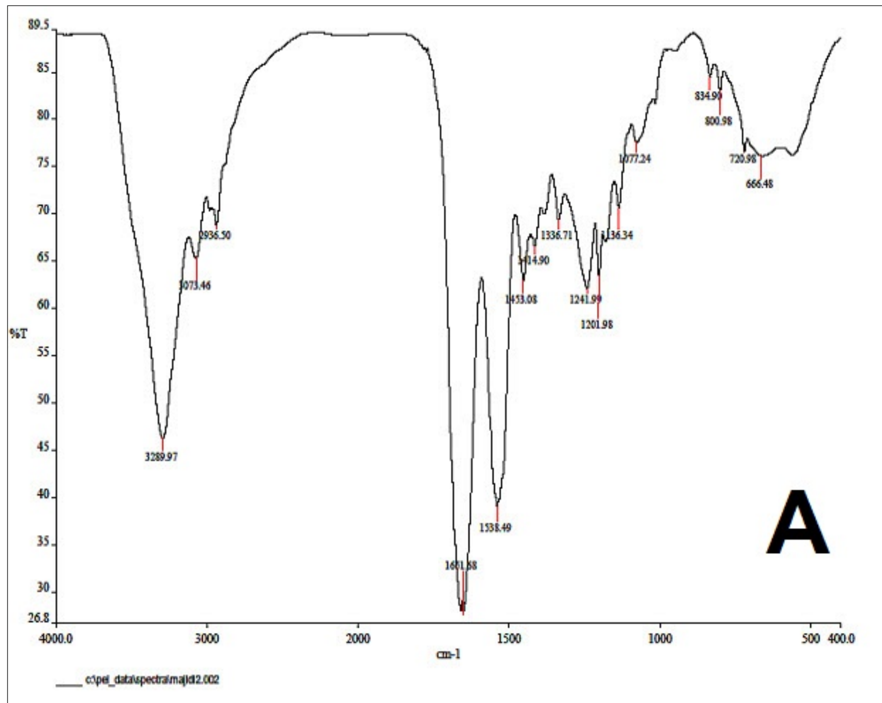

Chart A. Silk fibroin – gelatin scaffold

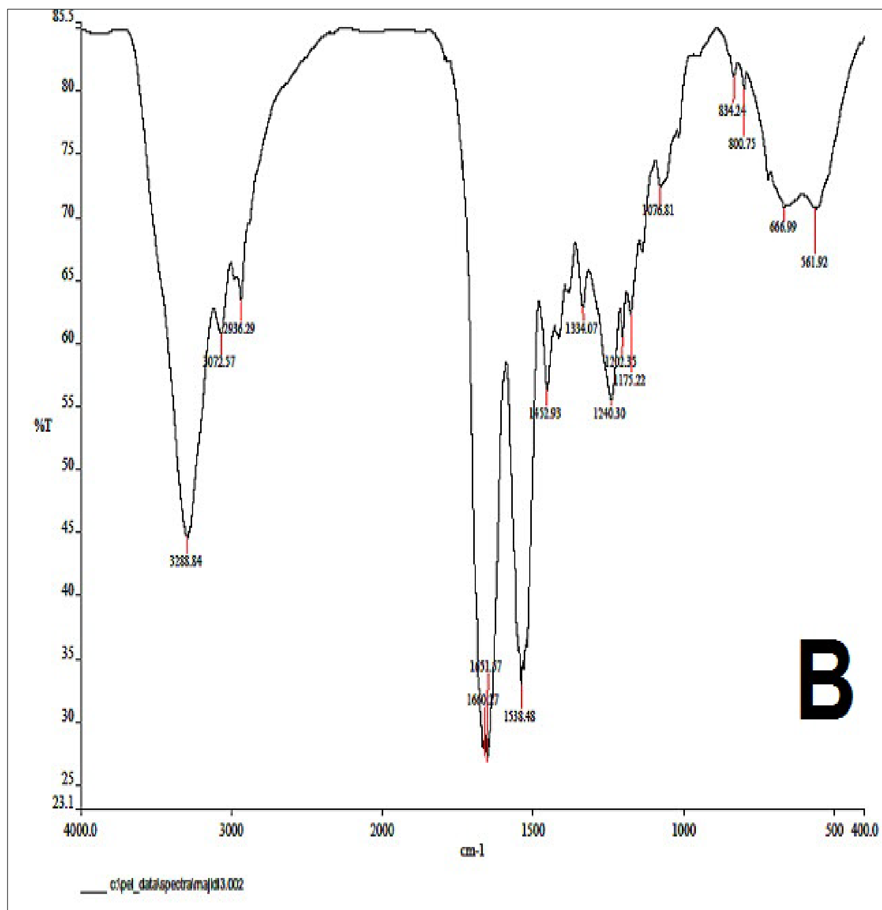

Chart B. Silk fibroin - gelatin - polyvinyl alcohol scaffold

Review of the results of the tests carried out on the cells

Results of scanning electron microscopy:

As seen in (Figures A, B), the scaffolds support the connection of chondrocyte cells and they have created a bed to so that chondrocyte cells of both scaffolds grow, maintain their phenotype, and start to secrete extracellular proteins and connect to the scaffolds and create a mesh of chondrocyte cells.

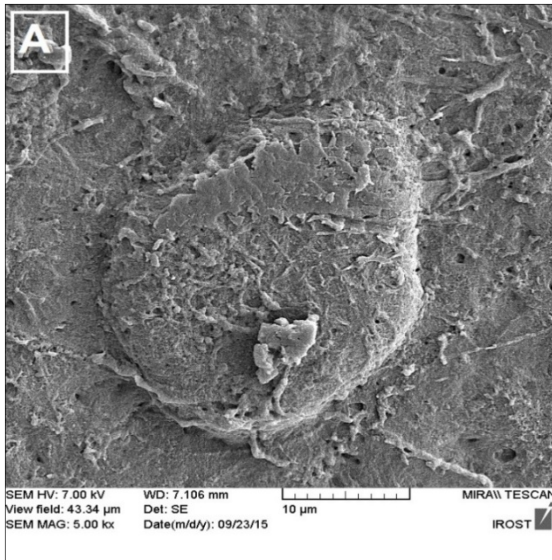

Figure A. growth of cells on silicone fibroin – gelatin – Poly vinyl alcohol scaffold

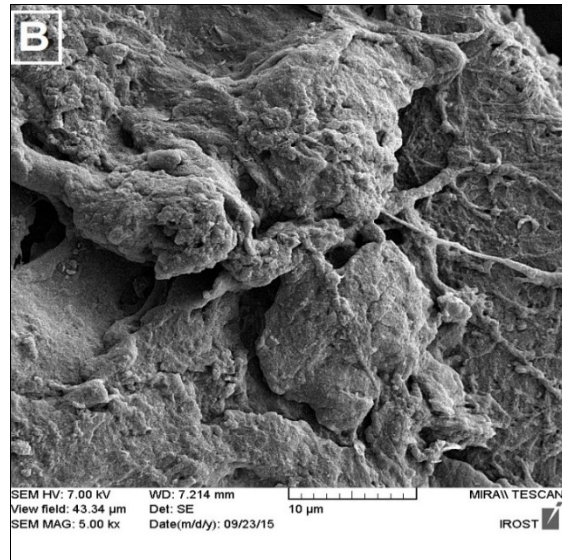

Figure B. Growth of cells on silk-fibroin gelatin scaffolds

Investigations deriving from results of silk fibroin-gelatin scaffold with silk fibroin-gelatin-poly vinyl alcohol scaffold:

In investigating the result of electron microscope, the diameter of the silk fibroin-gelatin scaffold is 57.59 nm the diameter of silk fibroin-gelatin-poly vinyl alcohol scaffold is 155 nm. Thus, the diameter of the silk fibroin-gelatin scaffold is better. In investigation of results related to tensile strength of two scaffolds, the silk fibroin-gelatin scaffold tolerates more tensile strength and has higher tensile strength. In investigations of results related to growth of the cell on two scaffolds, more cells grew on the silk fibroin-gelatin scaffold, and the rate of secretion of glycosaminoglycan was higher, indicating that silk fibroin-gelatin scaffold has provided better conditions for growth of cells.

Investigating the result of the biodegradability of scaffolds revealed that the silk gelatin-poly vinyl alcohol scaffold was degraded less than its structure after three weeks, which is due to the presence of a poly vinyl alcohol polymer that is a synthetic polymer and has lower rate of degradation compared to natural polymers. The presence of this polymer in the structure of this scaffold has resulted in slower rate of degradation.

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