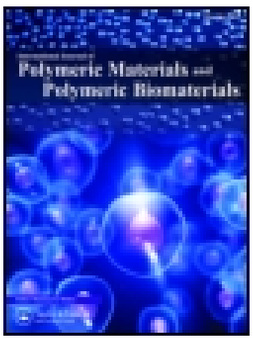

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Novel 3D Electrospun Polyamide Scaffolds Prepared by 3D Printed Collectors and their Interaction with Chondrocytes

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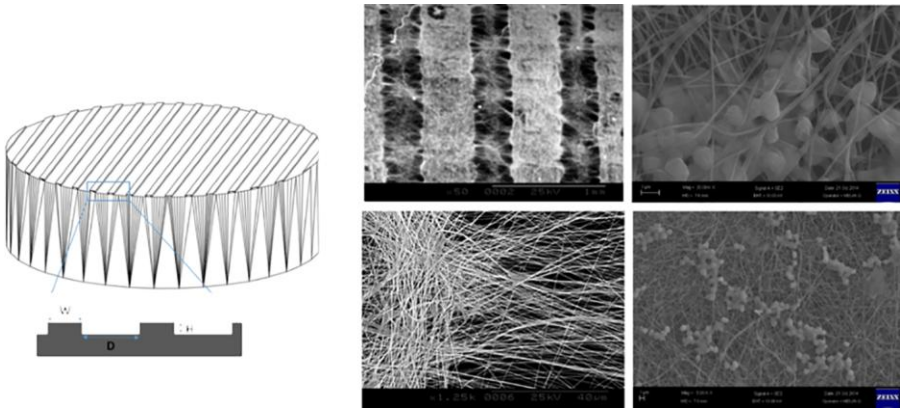
Abstract

Electrospinning is significantly one of the simple and versatile methods for producing micro- and nanofibrous scaffolds. Its assembly can be modified in different ways to combine material properties with different morphological structures for biomedical applications. In this process, collector design plays an important role to determine the nanofibers orientation in electrospun nanoweb. In this work 3D patterned scaffolds were produced by electrospinning of Polyamide-66 solution on different 3D collectors which have been obtained. The aim of this work is to investigate the attachment of the chondrocyte cells on the prepared electrospun scaffolds which have different type of nanofibers orientations that could be used in tissue engineering applications.

GRAPHICAL ABSTRACT:



KEYWORDS: Electrospinning, patterned collectors, 3D printer, Polyamide-66 scaffold, tissue engineering, cell culture, chondrocytes, nanofibers

1. INTRODUCTION

Electrospinning (ES) is certainly the one of the most versatile methods to prepare ultra-fine nanofibers to the order of few nanometers. In this process, a high electric field charges positively the droplet of polymer solution coming out from the tip of spinneret. The ejected jet from the nozzle deposit the nanofibers over a grounded metallic collector [1].

Nanofibers, produced by ES technique, have been explored for their unique properties (high surface to weight ratio, highly porous mesh) in various fields such as biomedical, filtration, fiber-based sensors, etc. [2-4]. Electrospun nanofibers have received much attention in biomedical application due to their similarity with collagen fibers in extracellular matrix (ECM), large surface area to volume ratio, high porosity and etc. [5, 6]

Nano-fibrous materials are good candidates for this kind of application and fiber orientation plays an important role in cell attachment and proliferation [7]. Typically, in an electrospinning process, nanofibers are disposed on a flat collector randomly oriented; however, in some applications, especially in tissue engineering well-aligned nanofibers in an electrospun scaffold is needed. Collector design and composition are the most crucial parameters of electrospinning process which affects the structure and orientation of nanofibers in electrospun nanoweb. Several approaches with different collector type are studied in the recent works such as static (plate, electrodes) and dynamic collectors (rotating disc and drum). [8]

By manipulating the electrostatic forces through specially designed collector or collecting the electrospun jet onto a rapidly rotating collector, nanofibers' arrangement can be changed from random to highly oriented. [9, 10]

Recently, it has been proven that 3D patterned scaffolds with different nanofiber's orientations and alignments can provide a structure able to mimic the natural ECM environment. Rogers et al, have presented a method to produce 3D collectors with various 3D geometries by using rapid prototyping method. Afterwards, 3D patterned sinusoidal PLGA electrospun scaffold with random and controlled 3D micro-patterns were produced. In the next step, they have evaluated the influence of produced patterned scaffold on 3T3 fibroblasts cell attachment and proliferation in vitro. It has been reported that fibroblasts were adhered to the aligned PLGA microfiber of the sinusoidal waves with no cell penetration. [11, 12]

Subramanian et al have reported the use of rotating mandrel as a collector in electrospinning machine. For achieving maximum orientation of nanofibers, the speed of rotating mandrel was varied from 1000 to 2500 rpm by keeping all the other parameters constant. They have observed that by increasing the speed above 2000 rpm, fiber breakages will appear. It has been shown that highly aligned electrospun nanofibers may promote significant progress in cell attachment and proliferation in the case of neural cells in nerve regeneration. [13, 14]

Although, these methods have the ability to control and produce aligned electrospun nanofibers but they have some defects such as long production time and imprecision. That is way, this study was carried out to develop a new method which assure fast, precise and controllable production of 3D collectors. [15]

The main objective of this work is to combine 3D printing technology and electrospinning process, as a novel method, to produce highly oriented and randomly oriented nanofibers within the 3D patterned electrospun scaffolds. According to literature, except one study of Rogers et al. which utilized patterned resin collectors produced by the additive manufacturing (AM) approaches during electrospinning, there is no other study. [11]

Different 3D collectors which have been obtained by 3D printing technique were used in electrospinning system to produce Polyamide-66 (PA-66) scaffold. We proposed a design of the frames to be produced by the 3D printer to facilitate the scaffold characterization.

Cells have different growth behavior over an electrospun nanoweb scaffold depending on nanofibers' orientation. The efficiency of this method was validated by performing a cell culture test using Chondrocyte cells. It was expected that the biological experiments in this study could indicate the ability of the PA-66 3D scaffold to support cell activities.

2. MATERIALS AND METHODS

2.1 Mechanical Parts Using 3D Printing

3D printing or AM is one of the rapid prototyping systems to produce a 3D object of almost any shape from a 3D CAD model. In this work, PolyJet Direct 3D printing (Objet Eden 260V, France) was used. The models of frames and different groove size collectors were designed by AutoCAD software, then uploaded into 3D printer machine for fabrication. Afterwards, the collectors were observed and surface and also side view images were taken by SEM.

The process of 3D printing is as following: first, the placement of resin on the build tray is calculated automatically by the machine software from the given CAD model. Then, the printer jets a curable liquid photopolymer and instantly cures the resin. Subsequently, the fine layers of resin shape the 3D model. If it is needed, it also jets a gel-like support material to support complicated geometries at the same time. The support materials can be easily removed by hand or water jet and no post-curing is needed [15]. To characterize the produced electrospun scaffold after electrospinning, supporting frames were fabricated in order to maintain the nanowebs fixed and to avoid any deformation of structure during characterization methods. The frames are used as a carrier to facilitate

sample handling for biological experiment. The frames with 1.5 cm circle hole in the center and 2mm thickness are composed of 3 parts; upper frame, base frame and lock, as it is presented in Figure 2. The frames can be tied by using 4 plugs and slots, and a lock can be put under the base frame to keep the collector tightly. Actually, the lock has one more important usage, which helps to pull the collector out from the frames after the electrospinning with minimal deformation of nanowebs.

Fabrication of Micro Patterned Collector

6 collectors having different patterns were produced to investigate the effect of collector geometry on nanofibers orientation and architecture (Figure 1). In this way, 6 CAD models of patterned collectors with different groove's dimension and constant height (H) of 0.5 mm were uploaded into the 3D printer machine, as shown in table 1.

Collectors were observed by Scanning Electron Microscope (Quanta 400F Field Emission, USA) of 50kV and surface and side view photos of collectors were recorded and analyzed. All collectors were coated with a thin layer of gold to be conductive and allow fiber deposition.

2.2 3D Scaffold Production

Polymer and Solution

PA-66 was selected because of its good mechanical and physical properties. Moreover, it has been proven that PA-66 has good biocompatibility with human tissue [1, 16]. Commercially available Polyamide-66 (Acros Organics, USA) was dissolved in formic

acid (98%, purchased from Fisher Scientific, France) to produce polymeric solution with four different concentrations; 15, 17, 20 and 25 wt.%. Then, stirring was applied by using a magnetic stirrer for 24h at 53ºC.

Electrospinning

In this study a home-made electrospinning machine with single nozzle was used. This device allows producing nanofibers with diameters between 50 and 500 nm. The device consists of an automatic pump, feeding Teflon tube, spinning needle, voltage system and collector plate.

The prepared solution was injected through a metallic needle with gauge of 0.7 mm in diameter and 30 mm in length by means of an automatic pump placed outside of the cabin. The polymer solution and applied voltage were 0.119 mL/h and 30 kV respectively. Although the mentioned above parameters were kept constant, electrospinning distance was varied from 10 to 30.

As it is presented in Figure 3 (A-F), collectors were put into the base frame (3B) and then both of them were placed over an aluminum foil inside the electrospinning machine. Once polymer solution was electrospun over collector surface (3C), upper frame was put on the base frame and the frames were tied (3D). The collector was pulled out carefully from the frames by lock (3E, F) and the electrospun scaffold remained fixed between upper and base frame.

Characterization of Electrospun Nanofibers

Morphology of the patterned electrospun scaffolds were visualized by SEM machine (Hitachi S-2360). The images of SEM were analyzed by ImageJ® software to measure the fibers' diameter. Thirty individual nanofibers diameters were measured and the average diameters of nanofibers and standard deviation were calculated for each condition. Cell attachment to the scaffold and fluorescent labeling of the cells were also observed and photographed by using inverted phase-contrast fluorescence microscope (Nikon Eclipse Ti Nikon Instrument, Japan).

2.3. In Vitro Tests

The electrospun PA-66 scaffold with concentration of 17wt.% was selected for the in vitro test. It was a circular sample having a diameter of 1.5 cm. For the next step, the ability of the PA-66 electrospun 3D scaffold to support cell activities, specifically, the cellular viability, proliferation and differentiation was investigated. Chondrocyte cells were seeded on the electrospun scaffold with the grooved pattern. After cell labeling, with cell culture duration of 72 hours, the scaffolds were characterized by inverted microscope and the phase contrast images were taken.

Cell Culture

Frozen P2 (2^{nd} passage) chondrocytes were provided from histology and embryology department's cell reservoir (Department of Histology and Embryology, Faculty of Medicine, Mersin University, Mersin, Turkey). The cells were thawed and cultured in T25 flasks consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% (v/v) Fetal Bovine Serum (FBS;), 1% penicilline-streptomycine, 1% amphotericine-B and 2.5% L-Glutamine. Cell culture was performed at 37°C in 5% CO2 and in a humidified (95% RH) incubator. Culture media were changed every 3 days to achieve the efficient population of cells, as measured by an inverted microscope. Cells obtained at passages 2 were used in this study.

Cell Counting

Cultured chondrocytes were detached with 0.25 (v/v) % Trypsin-EDTA (Lonza) from T25 cell culture flasks. Harvested cells were counted with an automated cell counter system (Cedex XS- INNOVATIS) based on the Trypan Blue method.

Scaffold Sterilization and Cell Seeding

After optimization of the cell culture conditions, we selected the group which has "medium groove sizes"- table 1- to evaluate the first interaction of chondrocytes with the novel 3D electrospun scaffold. They were sterilized by immersing the structure in the solution of ethanol/water (70:30, v/v) for 2 hours and pre-wetted with DMEM before cell seeding in the laminar flow cabinet.

Considering the recent studies [1, 16, 17], a population of 8×10^4 cells was seeded on each scaffolds and also control in 6 well plates (5.3×10^4 per cm^2). Then, 3 mL supplemented medium was added to each well. For demonstrating cell proliferation, Bromodeoxyuridine (BrDU), a thymidine analog, was added to the medium in 10 µM

concentration. BrdU in culture is incorporated into DNA during DNA synthesis prior to cell dividing.

Cell Labeling

After 72 hours, attached cells were observed and photographed under the inverted phase-contrast photomicroscope. Scaffolds were washed 3 times with Phosphate Buffered Saline (PBS) solution. To fix the cells, 4% paraformaldehyde + 0.1 % detergent (Triton X-100), were added to each scaffolds for 30 minutes. This fixation method gives the best results for labeling the cells by fluorochrome-conjugated antibodies.

For observing divided cells, scaffolds were treated overnight with 1:300 diluted mouse monoclonal anti BrdU primary antibody (Novocastra Cat# NCL-BrdU) at 4 ^{0}C. Following the washing steps, 1:500 diluted Alexa-488 conjugated anti mouse secondary antibody (abcam, Cat# ab150113) were used for making visible the labeling. At the end, DAPI solution was prepared freshly and added for counter stain of nuclei to be observed under a fluorescence microscope. Observing and photographing were performed by the same microscope mentioned above.

3. RESULTS

3.1. Collectors Production

As it can be seen in Figure 4, the obtained results show that precision in the dimension of collectors was in accordance with specification of manufacture. It can be concluded that

3D printer technology allows to produce repeatable 3D patterned collectors with high precision in a short time.

3.2 Scaffold Fabrication

Figure 5 shows the result of nanofibers' diameter versus polymer solution concentration and needle tip to collector distance. According to the nanofibers characterization, as shown below, a condition of 17% (w/w) concentration with 30 cm needle-collector distance was selected. These conditions and parameters were chosen according to the smallest standard deviation (SD) and the smallest diameter of nanofibers.

SEM and inverted phase contrast microscope's images presented in Figure 6, 7 and 8 indicate the morphological properties of nanofibers and their structure in the produced scaffold. The images clearly presented the oriented and non-oriented parts in the scaffold. The results show that structured collectors allow producing nanowebs templates made of nanofibers with alternative pattern of oriented and non-oriented area.

According to these images, it can be included that by increasing the distance between the grooves with the constant groove' width in each group, the orientation parts do not increase as it was expected. By increasing the distance between the grooves, since the nanofibers cannot be stretched completely through the width the grooves, a non-oriented part appears between. Therefore, two narrow parts of oriented nanofibers on the edge can be seen.

3.3 Cell Proliferation, Morphology And Distribution

Representative images of labeled chondrocytes with DAPI and BrdU on the surface of patterned scaffold can be seen in Figure 9 (A, B). Fluorescence microscopy shows that chondrocytes had proliferation over the 3D PA-66 scaffold after 72 hours of culture. Besides previous characterization, the obtained images from fluorescent microscopy revealed a good chondrocyte attachment and penetration on the PA-66 patterned scaffold (Figure 9 (C, E). The obtained images from SEM also revealed a good chondrocyte attachment and penetration on the PA-66 patterned scaffold (Figure 9 (C, D)). In Figure 9 (C, D), in which the SEM image is taken from high magnification, it was obvious how the cells penetrated within the nanofibers. In Figure 10, the phase contrast image of the electrospun PA-66 scaffold after 72h of cell culture taken by inverted microscope is presented. The cells nearly reached to confluence on both the oriented and non-oriented parts of the scaffold. It is possible to mention that the confluence was higher on the oriented part. The orientation significantly affected the direction of the cells. The cells seemed to be directed parallel to the oriented nanofibers. The chondrocytes showed elliptical and elongated geometrical shape in both oriented and non-oriented parts.

4. DISCUSSION

As it was mentioned previously, by changing the design of the collector in the electrospinning process, a different rate of alignment and orientation of the electrospun nanofibers can be achieved. In the recent studies, researcher proposed a new fibers collection method [18]. For example, Vaquette had investigated the effect of patterned collector on the pore size of the electrospun nanofibers and highlighted high penetration

up to 250 μm of fibroblast cells into the scaffold [19]. However, produced 3D collectors using 3D printer technology in comparison to other methods, clearly proved some advantage such as fine detail, smooth surface, high speed and precision and no need of post-curing.

In this study, 3D collectors with different groove size patterns were fabricated by 3D printer. To facilitate the characterization of these patterned scaffolds, a pair of frames was produced. Afterward, PA-66 was electrospun onto the patterned collectors. Electrospinning with four mentioned concentrations was performed on the fabricated patterned collectors and detachments of the electrospun nanofibrous matrices from the collector with different concentration were observed.

After characterization by SEM and phase-contrast microscope (Figure 6 and 7), the findings clearly shows that, the PA-66 nanofibers are affected by both the geometries and dimensions of the fabricated 3D collectors. The random nanofibers were found mostly on the top of the grooves and on the edge, but the oriented nanofibers were seen in the valleys.

For the next step, Chondrocytes cells were counted and population of 8×10^4 chondrocyte cells were seeded on each samples and control plate. Then, they were placed in 6-well culture plates. The medium was added to each well-plate and also control to provide nutrition for keeping the cells alive.

On the other hand, by observing the labeled cells using the fluorescent microscopy, good attachment and proliferation of the chondrocyte cell on the PA-66 could be seen. SEM images revealed the attachment and penetration of the cells within the nanofibers (Figure 9 C, D); and migration and spreading of the cell groups on the surface of the scaffold (Figure 9 E, F, G). It was demonstrated previously that, since the structure of the nanofibers mimic very well the natural extracellular matrix, the electrospun nanofibrous matrices create a favorable environment for cells to attach and penetrate. In Figure 9 E, F and G, it is possible to see the overall structure from a lower magnification, where the cell groups spread on the surface of the scaffold. It was concluded that the nutrient transport was adequate and the surface was favorable for chondrocytes to attach, survive and proliferate. The viability and attachment of the cells were also supported by the result of microscopic observations after DAPI and BrdU cell labeling.

Figure 10 demonstrated the phase contrast image of the 3D PA-66 electrospun scaffold cultured with chondrocytes for 72 hours. The cells reached to higher confluence in the oriented part of the scaffold compared to non-oriented part. Moreover, it was very obvious that the cells were parallel to the direction of the oriented nanofibers. In the non-oriented part, the presence of cell groups directed in different paths were observed. In previous studies, it was demonstrated that cells showed different behavior depending on the structure of the scaffold. In this study, it was demonstrated that different orientations can be obtained by combining electrospinning and 3D printed collectors and as a result of cell interaction studies, the direction of the cells could be tailored. In addition, the confluence of the cells on the scaffold showed the biocompatibility of the matrices. The

structure of the scaffold allowed transport of nutrients and wastes, therefore the cells tended to proliferate and migrate on the nanofibrous matrices.

5. CONCLUSION

This work, had investigated the potential of 3D printing technology when it is combined with the electrospinning method to produce 3D scaffolds in nanoscale. In this study, six patterned collectors with different groove sizes have been produced by using 3D printer. The polyamide-66 nanofibers were produced on the patterned collectors by electrospinning. The characterization results have shown that the PA-66 nanofibers follow both the geometries and dimensions of the fabricated 3D collectors. Thus, it is possible to produce electrospun scaffolds with repeatable 3D geometry. It has been demonstrated also that structured collectors allow producing nanowebs templates made of nanofibers with an alternative pattern of oriented and non-oriented areas, which can provide an appropriate surface for cell growth. In order to evaluate the in vitro performances of the produced scaffolds, chondrocytes were seeded onto the scaffolds and cell attachment and proliferation abilities were evaluated on different parts of the patterned scaffolds. The cells penetrated inside and within the nanofibers, while they spread on the overall surface. The viability and proliferation of the cells were proven by microscopic analysis after cell labeling by DAPI and BrdU. The chondrocytes showed elliptical and elongated shapes on the nanofibers and reached to near confluence on the novel 3D electrospun nanofibrous matrices. The orientation of the nanofibers guided the direction of the cells. The overall results demonstrated the possibility to prepare 3D differently structured electrospun nanofibrous mats by using 3D printing technology.

Since, the fabricated scaffolds provided a favorable environment for the chondrocytes to attach, proliferate and migrate and they enable us to control the direction of the cells, electrospun 3D nanofibrous matrices can be potentially used in tissue engineering applications.

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Table 1. The different dimension of grooves on the collectors

Samples Grooves	Width (W) [mm]	Distance Between Grooves (D) [mm]
Small (S1)	0.2	0.4
Small (S2)	0.2	0.6
Small (S3)	0.2	0.8
Medium (M1)	0.4	0.4
Medium (M2)	0.4	0.6
Medium (M3)	0.4	0.8

Figure 1. CAD model of collector with grooves

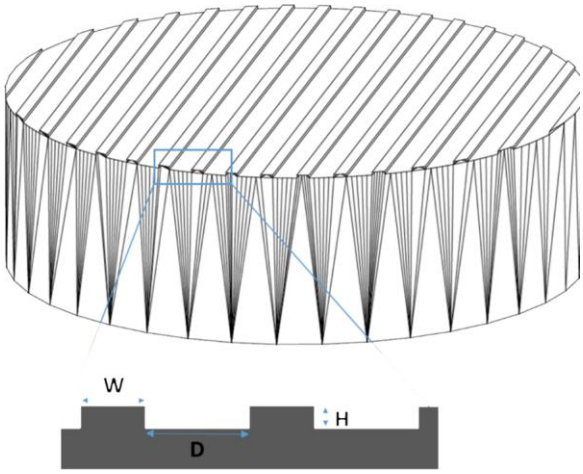


Figure 2. Different parts of fabricated frames with a collector

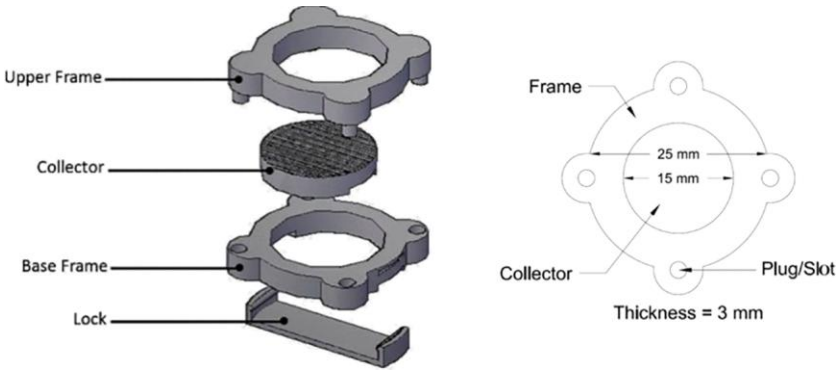


Figure 3. The process of electrospinning on the 3D collector placed between the frames. (A) Base frame, (B) collector placed in the base frame, (C) electrospinning on the patterned collector, (D) upper frame, (E) pulling out the collector, (F) produced patterned scaffold

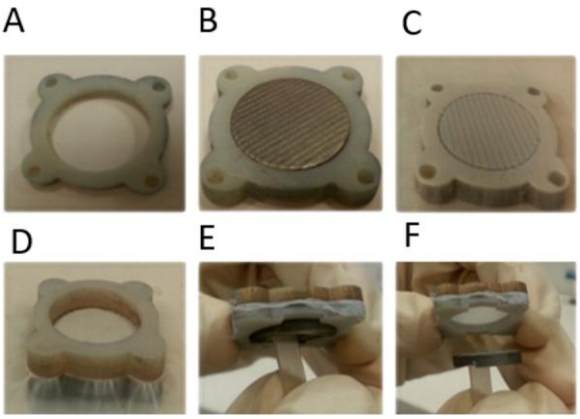


Figure 4. (A)(C) SEM image of the collectors S1 and M2, (B) SEM image showing side view of the 3D printed collectors S1 and M2

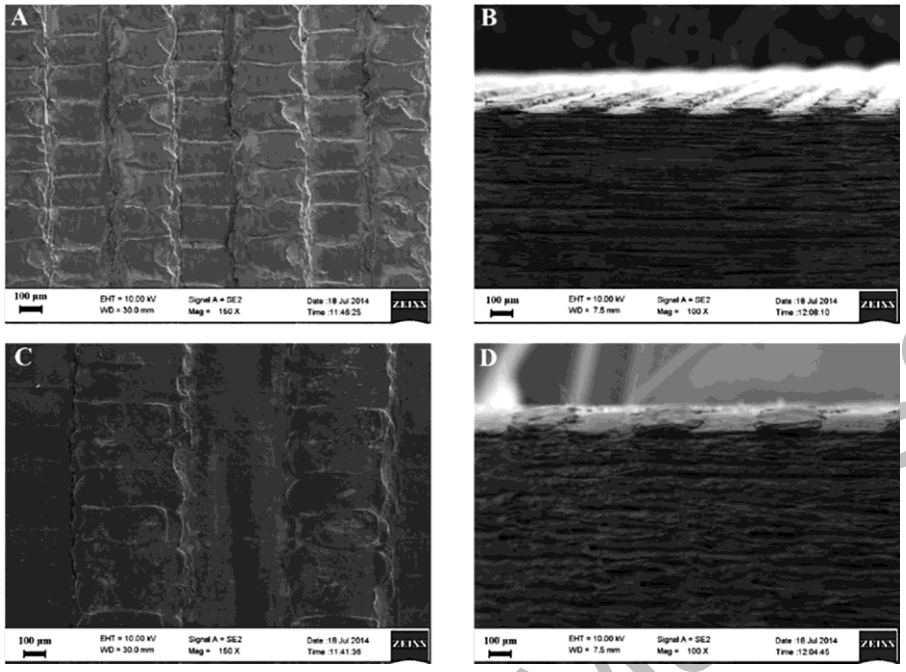


Figure 5. Selected condition for electrospinning

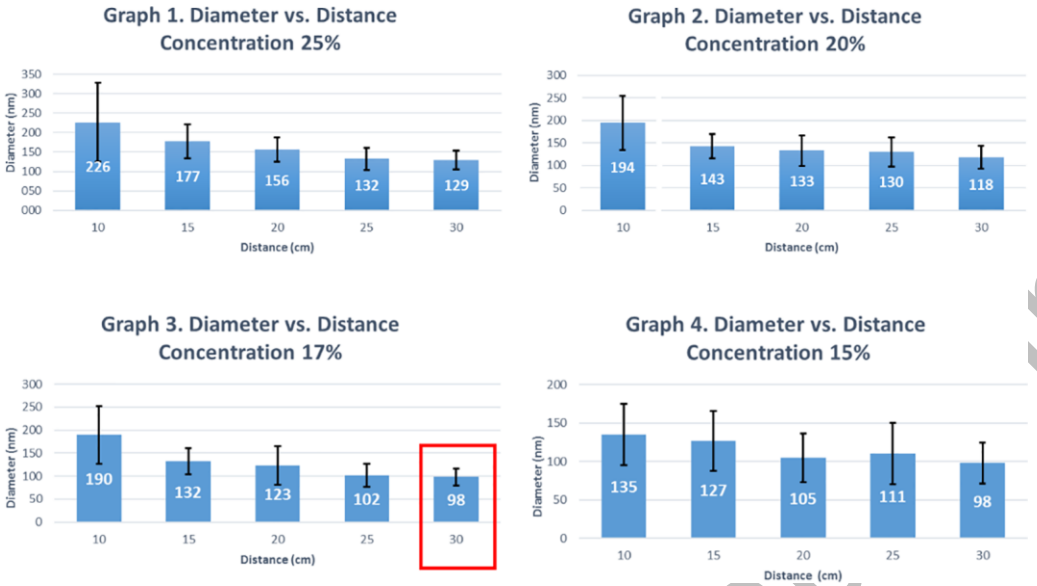


Figure 6. SEM images of PA-66 (17%w/w and electrospinning distance of 30 cm) nanoweb from different patterned collectors. (A)S1, (B) S2, (C) S3, (D) M1, (E) M2, (F) M3

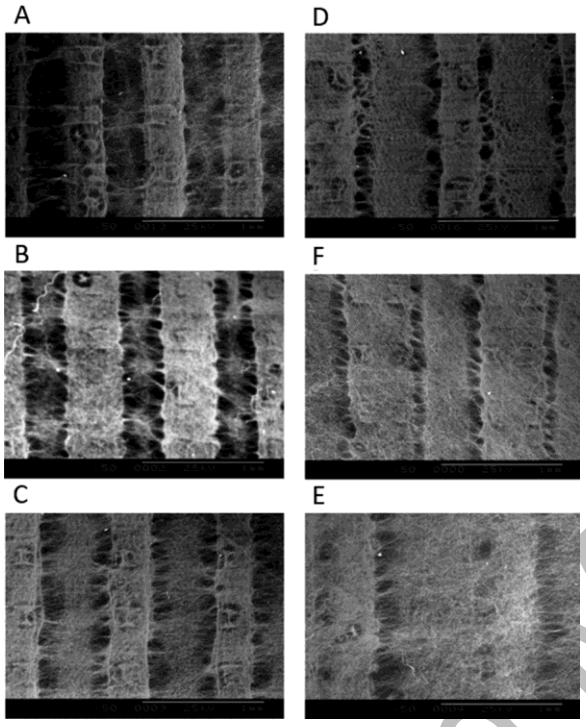


Figure 7. SEM images of PA-66 (17%w/w electrospinning distance of 30 cm) nanoweb using collector S1. (A, B) Oriented and non-oriented parts of scaffold, (C) oriented nanofibers, (D) non-oriented nanofibers

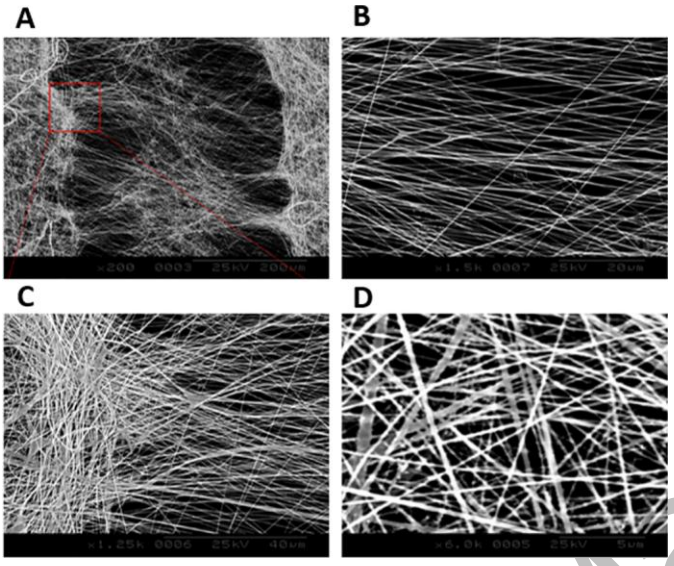
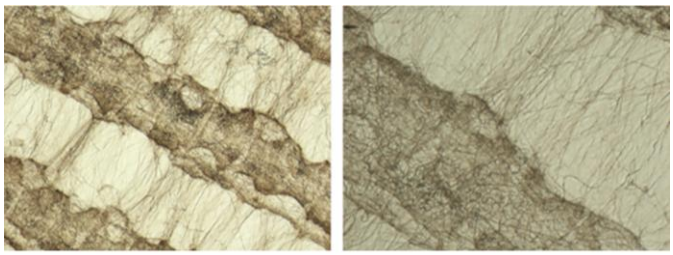


Figure 8. Image of scaffolds S1 (17%w/w), (A) magnification x10 and (B) magnification x20, taken with inverted phase-contrast microscope



(a) (b)

Figure 9. Observed labelled Chondrocyte cells with BrDU - M3 (A) and DAPI - M3 (B) on the patterned scaffold by inverted microscopy after 72h of culture. (C, D, E, F) SEM images showing the attached cells on the PA-66 (17%w/w) electrospun scaffold – samples M1

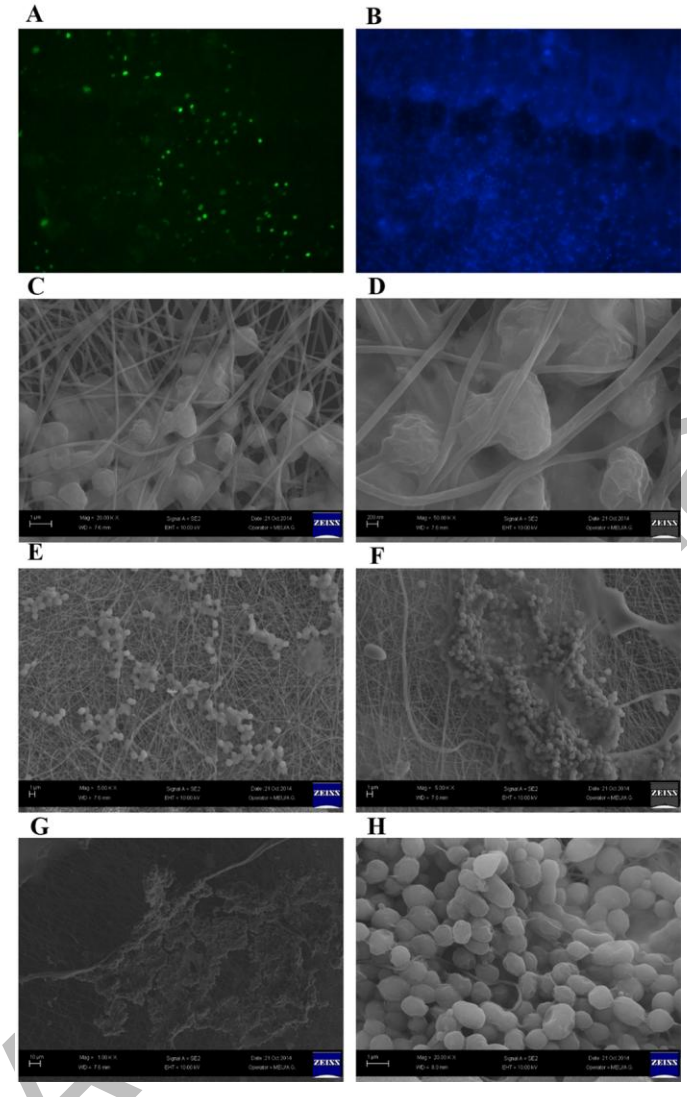


Figure 10. The phase contrast image after 72h of cell culture taken by inverted microscope - sample M1, magnification X10