Design and Characterization of Biodegradable Multi Layered Electrospun Nanofibers for Corneal Tissue Engineering Applications

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Abstract

Tissue engineering is one of the most promising areas for treatment of various ophthalmic diseases particularly for patients who suffer from limbal stem cell deficiency (LSCD) and this is due to the lack of existence of appropriate matrix for stem cell regeneration. The aim of this research project is to design and fabricate triple layered electrospun nanofibers as a suitable corneal tissue engineering scaffold and the objective is to investigate and perform various in–vitro tests to find the most optimum and suitable scaffold for this purpose.

Electrospun scaffolds were prepared in three layers. Poly (D, L-lactide-co-glycolide; PLGA, 50:50) nanofibers were electrospun as outer and inner layers of the scaffold and aligned type I collagen nanofibers were electrospun in the middle layer. Furthermore, the scaffolds were crosslinked by 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Glutaraldehyde. Structural, physical, and mechanical properties of scaffolds were investigated by using N2 adsorption/desorption isotherms, Fourier transform infrared spectroscopy (FTIR), contact angle measurement, tensile test, degradation, shrinkage analysis, and scanning electron microscopy (SEM). Also, capability to support cell attachment and viability were characterized by SEM, MTT assay and DAPI staining. According to the result of BET analysis, specific surface area of electrospun scaffold was about 23.7 m2 g-1. Tensile tests on crosslinked scaffolds represented more suitable hydrophilicity and tensile behavior. In addition, degradation rate analysis indicated that non-crosslinked scaffolds degraded faster than crosslinked one and crosslinking led to controlled shrinkage in the scaffold. The SEM analysis depicted nano-sized fibers in good shape. Also, the *in-vitro* study represented an

improved cell attachment and proliferation in the presence of human endometrial stem cells (hEnSCs) for both crosslinked and non-crosslinked samples. The current study suggests the possibility of producing an appropriate substrate for successful cornea tissue engineering with a novel design.

Key Words: Electrospinning, Corneal, Tissue Engineering, Collagen, PLGA, Human Endometrial Stem Cells (hEnSCs)

Introduction:

According to the literature, more than 10 million people in the world suffer from corneal disorder [1]. Inflammation, infections, trauma, chemical burns, cancer, systemic diseases, and pathologic disorders of adjacent tissues, can ultimately cause visual impairment and even blindness [1].

Allogenic corneal transplantations are available in most countries as the first treatment option, but the immunological rejection, corneal donor deficiency and reducing the quality of donated corneal due to LASIK surgery remain unresolved challenges [2]. Therefore, many researchers have tried to find a method for regenerating the damaged cornea. Human amniotic membrane (HAM), is a popular natural material that widely used to restore corneal epithelial defect and is considered as a gold standard scaffold for corneal epithelial cells [3]. According to the research, HAM induced wound healing and possesses anti-inflammation and anti-fibrotic effects. However, biodiversity among donor tissues, unavailability, contamination and transmission of infectious diseases are some of the limitation and side effects of HAM [4].

Corneal tissue engineering is another method which can be effective for the treatment of corneal injury [1]. The first step in tissue engineering is designing appropriate scaffold with suitable physical property for proper cell and tissue support. In addition, the stiffness and transparency of scaffold equal to the native corneal tissue were the main issues for fabricating this structure in vitro which need to be considered [5]. Many methods have been investigated for bioengineering the scaffold to mimetic extracellular matrix systems for corneal tissue [6]. Designing of matrix systems by natural membranes, decellularized corneal tissue and prefabricated scaffold by polymers are some of these methods. These approaches also have some challenges, including inappropriate mechanical property and immunogenicity of decellularized systems [7]. Prefabricated 3-dimentional scaffolds by suitable polymer provides an applicable strategy to solve

these problems in corneal tissue engineering [8]. The Biodegradable scaffolds designed by nanofibers have used as a great structure for cell attachment and proliferation [9].

Natural and synthetic biomaterials were widely used in the past [10]. Natural polymers have excellent compatibility but poor mechanical and thermal properties while synthetic polymers can be optimized for desirable properties. Combination of synthetic and natural polymers leading to prevail these disadvantages [10]. Collagen is one of the most abundant materials within the human body that have excellent biocompatibility and low immunogenicity. Since the collagen is a major component of ECM and a vital ingredient in the corneal stroma, it can be an excellent approach to reconstruct corneal tissue, equivalents as human stromal tissue [11, 12]. However; the manipulating of collagen is hard, because of highly hydrated nature of the normal collagen gel. Several methods have been used previously to resolve the mechanical defects of collagen, for instance, using suitable cross-linker or combining them with other polymers, considerably improve its mechanical and chemical stability of collagen [13, 14]. For example, Polyhydroxy acid group is one of the most popular polymers which has been used recently in tissue engineering [15]. PLA (poly lactic acid), PGA (poly glycolic acid) and PLGA (lactide-glycolide copolymer) are located in this group, widely used in tissue engineering [16]. These destructive polymers are made up of two synthetic polymers PGA and PLA with different percentages which have been used for various applications in tissue engineering [17]. The advantage associated with these copolymers is designing and optimizing predictable degradation rate for various applications [14]. According to recent studies, this synthetic membrane could support the growth of the limbal cells and repair damaged corneal epithelial in the rabbit model and this kind of polymer can be stored for 12 months post gamma radiation, and it will, therefore, be readily available in the cornea surgery.

There are various methods existing for producing nanofibers. For the past few years, electrospinning technique has attracted scientists to produce nanofibers for various tissues

regeneration [18, 19]. Due to the structure, its provide a large, surface-to-volume ratio, that is favorable for cell attachment and proliferation, as well as excellent stability [20].

The aim of this study was to design and fabricate triple layered electrospun nanofibers PLGA - collagen - PLGA as an appropriate corneal tissue engineering scaffold. Collagen and PLGA were used to improve mechanical properties and biocompatibility of scaffold. Poly D, L-lactide-co-glycolide; PLGA (50:50) nanofibers were electrospun randomly, as outer and inner layers of scaffold in order to be used as a substrate for corneal epithelial and endothelial cells while aligned type I collagen nanofibers were electrospun in the middle layer as a substrate for corneal stroma. Aligned collagen fibrils shown to be significantly having a higher mechanical property rather than random form with enough stromal transparency which is due to small size and aligned collagen fibrils [21]. The shrinkage of electrospun PLGA is undesirable behavior in cell culture condition. Further means stability, swelling and mechanical properties of collagen can be tailored by variation of the crosslinking status. In order to control PLGA shrinkage and collagen behavior, the scaffolds were crosslinked by various concentration of 1-ethyl-3-(3 dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and Glutaraldehyde. Cell viability and cell activities were investigated by using Endometrial Stem Cells (hEnSCs), as an appropriate source of mesenchymal stem cells on the designed nanofiber scaffold and its potential for corneal replacement [22].

Materials and methed

Materials:

Poly D, L-lactide-co-glycolide;(PLGA) with 50:50 ratio of lactide and glycolide (Mw 44 kg/mol), type I collagen, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Glutaraldehyde, trypsin, phosphate-buffered saline (PBS), 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), collagenase I, and 4′,6-diamidino-2-phenylindole (DAPI), were prepared from Sigma-Aldrich. N'N-dimethylformamide (DMF), Chloroform, and glacial acetic acid were prepared from Merck. Dulbecco's Modified Eagle's Medium (DMEM-F12) was purchased from Invitrogen (Carlsbad, CA).

Construction of electrospunthree-layer scaffold:

PLGA was dissolved in Chloroform 90% and DMF 10% at a concentration of 17% (wt/wt) and stirred for 1 hour at room temperature. The solution was aspirated in a 5mL syringe with a needle gauge of 20 that attached to high voltage. Electrospinning performed with a flow rate between 0.5-1mL/h with a voltage between 15-20 kv. The electrospun fibers were collected on the collector that covered by an aluminum foil with rotational speed at 450 rpm. Collagen type I was dissolved in acetic acid (5%) at a concentration of 6% (wt/wt) and stirred for 2 hours at room temperature. Collagen was electrospun with the flow rate of 0.5-1mL/h with the voltage between 12 to 17 kV and rotational speed was at 1800 rpm to obtain aligned nanofibers. PLGA solution was electrospun on top of collagen as the third layer with the same condition explained previously. Finally, the multilayer scaffold was fabricated with a thickness between 500-600μm.

Cross-linking the scaffold:

In this study 2 crosslinker have been compared together. Scaffolds divided in3 groups and samples were prepared in dimensions 2 cm × 2 cm. First group cross-linked with glutaraldehyde 2.5% that was dissolved in ethanol 99% (Fisher Scientific, Inc.) at room temperature for 6 hours and second group cross-linked by 40 mM EDC/20-mM NHS that dissolved in ethanol 99% (Fisher Scientific, Inc.) for 1 hour at room temperature.

Characterization of designed scaffold:

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM; Philips XL30) was used to investigate the morphology of fabricated electrospun nanofibers and the attached cell at the voltage of 15 kV. Scaffold cut into pieces (0.5 cm × 0.5 cm) and placed on the holder, then the samples were gold coated using a Sputter coating (EMITECH K450X, England). The cell fixation was carried out 72 h after cell seeding by adding 2.5% glutaraldehyde on the cells and remaining for 2 h at room temperature. Dehydration was carried out using a series of ethanol concentrations (50%, 70%, 80%, 90%, and 96%) in distilled water for 10 min per each concentration and then freeze-drying for 24 h. The average pore size and diameter of the nanofibers were measured, and Image J was used to analyze nanofiber morphology (ImageJ; National Institutes of Health).

Brunauer Emmett Teller (BET) for Nitrogen Adsorption

The physical absorption of the nitrogen gas is one of the methods that can be used to measure the surface area, volume and porosity of scaffold with a diameter of less than 6 nm. In this study, the pores size measured by the amount of absorbed and desaturated gas relative pressure in this method, 500 µg of the scaffold was prepared with a dimension of 5 mm × 5 mm and exposed to nitrogen gas in order to calculate the surface area and measure the pore volume.

Mechanical properties

In order to determine the modulus and tensile strength, according to ASTM D882-02 (2002), three groups of scaffolds include, non-cross-linked, cross-linked with glutaraldehyde and cross-linked with EDC were examined using a universal testing machine (SANTAM). The rectangular form samples with dimension 10 mm × 30 mm were placed in the machine with a 500-N load cell at the cross-head speed of 1mm/min. For each group, 3 samples were examined and at least three samples were measured for each type of scaffold and finally, its average value was calculated.

Degradation rate

Measuring the amount of degradation as a percentage of weight loss is expressed in dry scaffolds. Three groups of scaffolds were merged in PBS and incubated at cell culture condition at various duration times (0, 14, 28, 42, 56, 70, 84 days). The scaffolds in three groups were weighed at the beginning of the test and then each scaffold was weighed weekly after removing from the medium. The scaffolds are dried using a vacuum machine before measurement. Losing weight is calculated and expressed as a percentage of the initial weight. The percent weight loss of the samples from the weight obtained before and after the destruction calculated using the following formula where W1 and W2 are the weight of the specimens before and after the degradation.

$$\text{Weight loss (\%)} = (W1-W2) / W1 \times 100$$

Contact angle

The study of the wettability of biomaterials is essential and can determine the degree of tendency for cell attachment and cell proliferation. Hydrophilicity of scaffold was determined by measuring the contact angle that analyzed by System (VCA Optima, AST products). The size of the drop was set in 0.5 ml. five samples were measured for every scaffold. And the mean value with SD (6SD) was reported.

Cell isolation and preparation

Human endometrial stem cells (hEnSCs) were isolated by enzymatical protocol as described previously [23]. Briefly, Endometrial tissue after chopping in 2.5 mg/ml collagenase I was incubated in DMEM-F12 medium at 37 °C for 45 minutes. Then, it was placed in a phosphate buffer saline (PBS) and the solution was centrifuged for about 5 minutes at 2000 RPM. After isolation, the cells were cultured in a flask with culture medium. The Culture medium that contains DMEM-F12 medium, 10% FBS and 1% penicillin/streptomycin were changed twice a week. After the third passage, the cells were prepared for cell identification and cell culture study.

Cell viability and attachment study

In this study for cell proliferation assessment, 4, 6-Diamidino-2- phenyl indole (DAPI) staining was used. Three groups of scaffolds were seeded by hEnSCs and incubated in culture medium within 96 well plates and maintained in a cell incubator for 3, 5, and 7 days. After cell fixation, adhesion, and proliferation of cells was assessed using DAPI staining. For cell cytotoxicity study, 3-(4, 5-dimethylthiazoyl-2-yl) 2, 5 diphenyl tetrazolium bromide (MTT) was used. MTT is one of the micro titration tests that examine the cell viability based on MTT revival. MTT is a water-soluble, and tetrazolium has a yellow color that is converted by living cells into a water-insoluble material in the form of purple crystals known as formazan. Formazan is dissolved in DMSO and measured by spectrophotometry with an ELIZA reader (Expert 96, Asys Hitch, Ec Austria) at a wavelength of 570 nm. To evaluate the cytotoxicity and viability of nanofiber scaffold, hEnSCs were seeded on the three groups of the scaffold at a concentration of 1×10^4 cells/well in 100 µl of culture medium in the 96 well plates, where each well was incubated for different time points. One sample was removed from the incubator 1, 3, 5, and 7 days after cell culture and cells were washed by PBS. 100 µL of MTT solution was added at a concentration of 0.5% to each plate and incubated for 4 hours. After removing the MTT solution, 100 µL DMSO was added in each well and incubated

for 10 minutes at room temperature and dark room. The absorbance was read by ELIZA reader and this test was repeated on days after seeding the cells on the scaffold.

Statistical Analysis

The results were statistically analyzed by GraphPad_Prism_8.0.1.244_x86 software using one-way ANOVA. The data were expressed as mean ± standard deviation (SD) of the means (n =3). In all evaluations, $P < 0.05$ was considered as statistically significant.

Results:

Assessment of electrospun nanofibers structures

SEM of electrospun nanofibers represent the morphology of scaffolds and estimate the average diameter pore size was about 1 – 10 µm in different magnifications. Figure 1(A and B), demonstrated the diameter of PLGA nanofibers that electrospun randomly with the range of 500-1000 nm. Figure 1(C and D) showed the nanofibers diameter were in the range of 200-300 nm and represent aligned electrospun nanofibers without presence of any bead, as well as a favorable space between the fibers, which can provide a good connection for the penetration of the cells. Figure 1 (E) showed the schematic designed corneal scaffold and figure 1 (E1, E2) overviewed the enlarged part of the electrospun nanofiber cross section of the overview of a scaffold with different magnifications. The effect of nanofibril network connection on cellular morphology was displayed by SEM in Fig. 8 after 7 days and 14 days and this figure indicated that cells were alive and attached significantly on nanofibers. These results showed these scaffolds can be used as a suitable substrate in appropriate conditions in tissue engineering.

Adsorption-desorption of nitrogen analysis

Figure 2 shows the N2 adsorption/desorption isotherms, surface area and cumulative pore volume of PLGA/Collagen/PLGA scaffold at 77 K. This result revealed a characteristic curve (type

III in standard IUPAC) without loop hysteresis and with a gentle slope at a relative pressure from 0.2 to 0.4, which called non-porous or microporous materials. The corresponding pore size distribution of the sample was calculated by the desorption branches of the isotherms using the BJH method (Barrett-Joyner-Halenda) while BET (Brunauer–Emmett–Teller) method was applied to the determination of samples surface area. The surface area of PLA/Collagen/PLGA scaffold is 23.7 m^2 g^{-1}. These results show the presence of woven fibers which represent relatively large holes between them. Finally, the collected results have a good compliance with other technique.

Degradation rate

The degradation results in this study (Fig. 3) showed that polymer degradation began after 7 days. After 49 days PLGA was degraded and absorbed completely, while after 49 days collagen is remained and absorbed gradually. Histogram (Fig. 3) showed a different degradation rate of cross-linked and non-cross-linked scaffolds. After 14 days the significant differences are found in the rate of degradation between the cross-linked scaffold and non-cross-linked scaffold however, there were not any significant differences seen ($p > 0.05$) between E and G group.

Mechanical property:

Table 1 indicated the mechanical properties of the designed scaffold with and without crosslinker. The typical stress-strain curves for the electrospun scaffold in 3 groups displayed. These results showed Young's Modulus (MPa), Tensile Strength (MPa) and percentage of Strain at Break which has been increased using EDC as a crosslinker. As results are shown in table 1, there are significant differences between the mechanical property of cross-linked and non-cross-linked scaffold ($P < 0.05$). According to the previous study, in natural corneal tissue, the tensile strength was 3–5 MPa and the strain at break was 0.192 [24]. Our results showed the modified scaffold by EDC would be appropriate for corneal tissue engineering (Fig. 4).

Contact angle:

Hydrophilicity assessment of PLGA/Collagen/PLGA nanofibers for cross-linked and non-cross-linked scaffolds after 10 seconds are shown in Fig. 5. According to this result, the contact angle was 97° before cross-liking and it has been reduced to 74° and 77° after cross-linked by EDC and Glutaraldehyde respectively. This reduction indicates the degree of nanofibers tendency for cell attachment and cell proliferation were increased after crosslinking.

Cell attachment and viability analysis

DAPI staining results (Fig. 6) indicated appropriate cells attachment as well as cell proliferation on PLGA/collagen/PLGA nanofibers for 3, 5, and 7 days. For cell metabolic activity study MTT assay was performed. The hEnSCs on the cross-linked and non-cross-linked PLGA/collagen/PLGA nanofibers, were investigated for viability at 3, 5, and 7 days (Fig. 7)

According to the results (Fig. 6, 7), there was more biocompatibility in cross-linked scaffold with EDC than other samples. MTT assay results performed 1, 3, 5 and 7 days after hEnSCs culture on PLGA-Collagen-PLGA nanofibers, and there was more cell viability in cross-linked scaffold than non-cross-linked samples. In consequence, although cells were attached and proliferated well on both cross-linked and non-cross-linked scaffolds but MTT assay certified higher biocompatibility in cross-linked scaffold than non-cross-linked.

Discussion:

Corneal tissue engineering is increasingly appearing as a powerful method for corneal defect treatment [1]. Designing an appropriate scaffold with suitable physical property for cell and tissue support is the first step for any tissue engineering application. In addition, the stiffness and transparency of scaffold equal to the native corneal tissue have been the main problems for fabricating this structure in vitro [5]. Engineering biodegradable nanofiber scaffolds can be the best approach to mimic a great structure for cell attachment and proliferation. Initially, this technique was designed for the textile industry [19]. Today, due to ability of this method to fabrication of woven webbing fibers, it is used as a suitable scaffold in tissue engineering. The

fibrous tissue produced by this method can imitate the normal shape and function of the extracellular matrix [18]. The limitation to forming scaffold and increase the thickness of the scaffold are some of the electrospinning challenges.

Given that corneal stroma contains aligned collagen fibers naturally, electrospinning of type I collagen is a suitable technique to achieve this structure; while random fibers can be useful to mimic epithelial and endothelial substrates [7]. Due to poor mechanical property and high hydrophilicity of collagen, it needs to be modified by appropriate cross linker that may effect on the cells that cultured on it. Furthermore, it can be improved by adding suitable synthetic polymer [25]. PLGA is one of the most well-known synthetic polymers to construct scaffolds for tissue engineering. The time of removal and absorption of this polymer is manageable and depend on location, shape, size, density, the ratio of monomers, molecular weight and morphology of copolymer [14].

In this study, 3-layers scaffold was fabricated using electrospinning of PLGA-Collagen-PLGA as sandwich nanofibers. PLGA solution (5ml) were spun randomly as a first layer, at concentration of 17% (wt/wt) after changing the voltage and distance between collector and needle in different tests, at 15-20 kV voltage, flow rate of 0.5-1mL/h and 450 rpm rotational speed which resulted in fabrication of nano size fibers. The primary layers of nanofibers were needed lower voltage than the end layers, because of the electrical conductivity was reduced by increasing the thickness of nanofibers on the collector. Following PLGA the second layer was fabricated using collagen solution (15ml) at a concentration of 6% (wt/wt), after optimizing the voltage and distance between collector and needle with different repeats. The parameters for electrospinning collagen include voltage between 12-17 kV voltages, the flow rate of 0.5-1mL/h and 1800 rpm rotational speed which resulted in the fabrication of aligned nanofibers. The third layer with PLGA was spun on aligned collagen nanofibers and the third layers completed with thickness between 500-600 µm thickness to mimic natural corneal thickness [26]. The major part of this thickness was aligned

collagen nanofibers. According to the research aligned electrospun fibers had high mechanical properties rather than random one at the same conditions. Furthermore, like natural corneal tissue, the aligned nanofiber scaffolds have less resistance to the light transmittance than random one [27].

In this study, the stability and mechanical properties of collagen and PLGA was one of the limitations. These materials modified by variation of crosslinking status. The scaffolds were divided into three groups and crosslinked by 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Glutaraldehyde. Two crosslinkers were compared together to determine suitable crosslinker to improve physical and mechanical property of scaffold. Cross-linking with glutaraldehyde caused a change in scaffold colour (yellowish) due to the formation of aldimine connection (CHaN) among the free amines of polymer and glutaraldehyde [25]. The morphology, mechanical properties, thermal, and biocompatibility of cross-linked and non-cross-linked scaffolds were characterized at the same condition. As shown in Fig. 8 all samples were suitable for cell attachment, while 14 days after cell seeding the structure of cross-linked samples especially EDC cross-linked, were more stable than the non-cross-linked scaffold. Also, the shrinkage of PLGA was controlled after cell culture (Fig. 8). According to the BET result, the specific surface area of nanofibers scaffold was about 23.7 $m^2 g^{-1}$.

Also, tensile tests in cross-linked scaffolds showed more suitable hydrophilicity and tensile behavior in cross-linked scaffold by EDC. In addition, degradation rate analysis indicated that non-crosslinked scaffolds degraded faster than cross-linked one. The selection of a suitable cell to reproduce target tissue plays an important role in tissue engineering. One of the sources of stem cells is Endometrial Stem Cells (hEnSCs) that contribute to the restoration of endometrial cycles [28]. These cells can be an appropriate source of mesenchymal stem cells because of its accessibility, easy cultivation [22], have anti-inflammatory properties and have a high degree of differentiation such as neuron cells, oligodendrocyte cells, adipocytes cells, osteoblastic cells and

beta cells of the pancreatic islet [29-30]. The specific cell markers such as CD44, CD146, CD90, and CD105 were expressed by these cells [23].

In vitro study showed that human endometrial stem cells (hEnSCs) were attached and proliferated well on both cross-linked and non-cross-linked samples but more cell viability in cross-linked scaffold than non-cross-linked. In addition, the transparency of scaffold increases by expands time of incubating in cell culture condition. This may due to gradually degrade of PLGA nanofibers. After cell culture, the shrinkage of PLGA was controlled by using EDC cross-linker.

Conclusions:

Designing an appropriate scaffold is essential for any tissue engineering product. Nanofiber scaffolds can be good approach due to the wide surface for cell contact and connectivity between macro-scale pores and appropriate mechanical properties. It was shown that mechanical and physical property of scaffold was highly relevant to the cross-linker. The swelling of collagen nanofibers, shrinkage of PLGA and degradation rate of PLGA-Collagen-PLGA nanofibers in cell culture condition were controlled after cross-linking with EDC. This study suggests the possibility of producing biodegradable electrospun nanofibers for corneal tissue engineering applications

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Figure legends:

Fig. 1: Scanning electron microscopy (SEM) with different magnifications (A and B) random electrospun PLGA nanofibers and (C and D) align electrospun Collagen nanofiber (E) schematic overview of designed corneal scaffold (E1, E2) cross section overwiew of scaffold with different magnifications.

Fig. 2: Adsorption-desorption of nitrogen analysis of PLGA/Collagen/PLGA nanofibers.

Fig. 3: Degradation rate analysis of PLGA/Collagen/PLGA nanofibers in 3 different groups. N: Non-cross-linked scaffold, E: Cross linked scaffold with EDC, G: Cross linked scaffold with Glutaraldehyde.

Fig. 4: The typical stress-strain curves for the electrospun scaffold in three groups. N: Scaffold without cross linker, E: Scaffold was cross-linked with EDC, G: Scaffold was cross-linked with Glutaraldehyde. Significant diff. among means ($P < 0.05$).

Fig. 5: Hydrophilicity assessment of PLGA/Collagen/PLGA nanofibers in three different groups. A: non-cross-linked scaffold, B: Scaffold cross-linked with Glutaraldehyde, C: Scaffold cross-linked with EDC.

Fig. 6: DAPI staining for hEnSCs on PLGA/Collagen/PLGA cross-linked and non-cross-linked scaffolds for 3, 5, and 7 days after cell culture.

Fig. 7: MTT analysis for hEnSCs on PLGA/Collagen/PLGA nanofibrous for 1, 3, 5, and 7 days after cell culture on. N: non-cross-linked scaffold, E: Scaffold cross linked with EDC, G: Scaffold cross linked with Glutaraldehyde.

Fig. 8: Scanning electron microscopy (SEM) after cell culture on cross-linked and non-cross-linked PLGA-Collagen-PLGA nanofibers scaffold at 7 and 14 days of seeding. N: non-cross-linked scaffold, E: Scaffold cross-linked with EDC, G: Scaffold cross-linked by Glutaraldehyde.

Figure 1

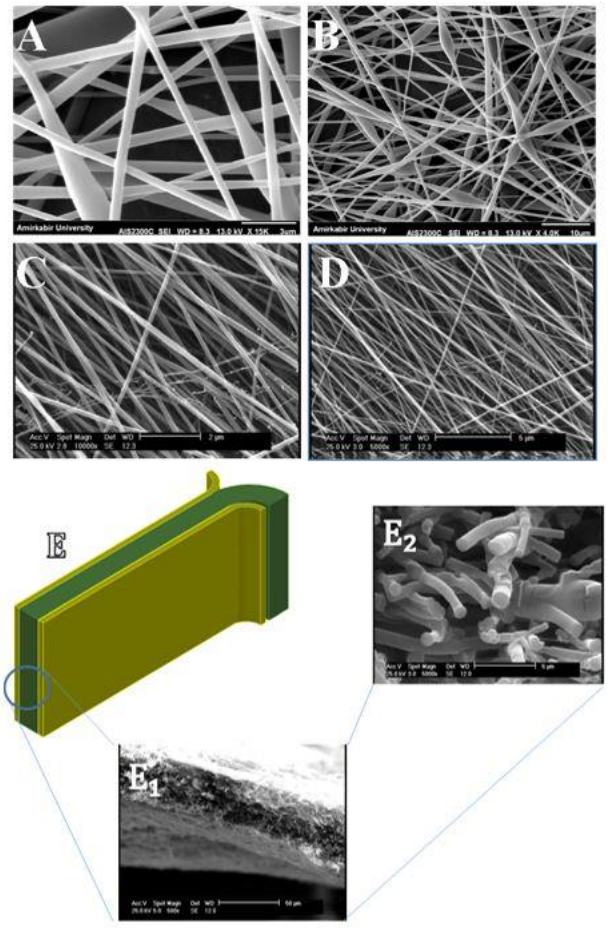

Figure 2

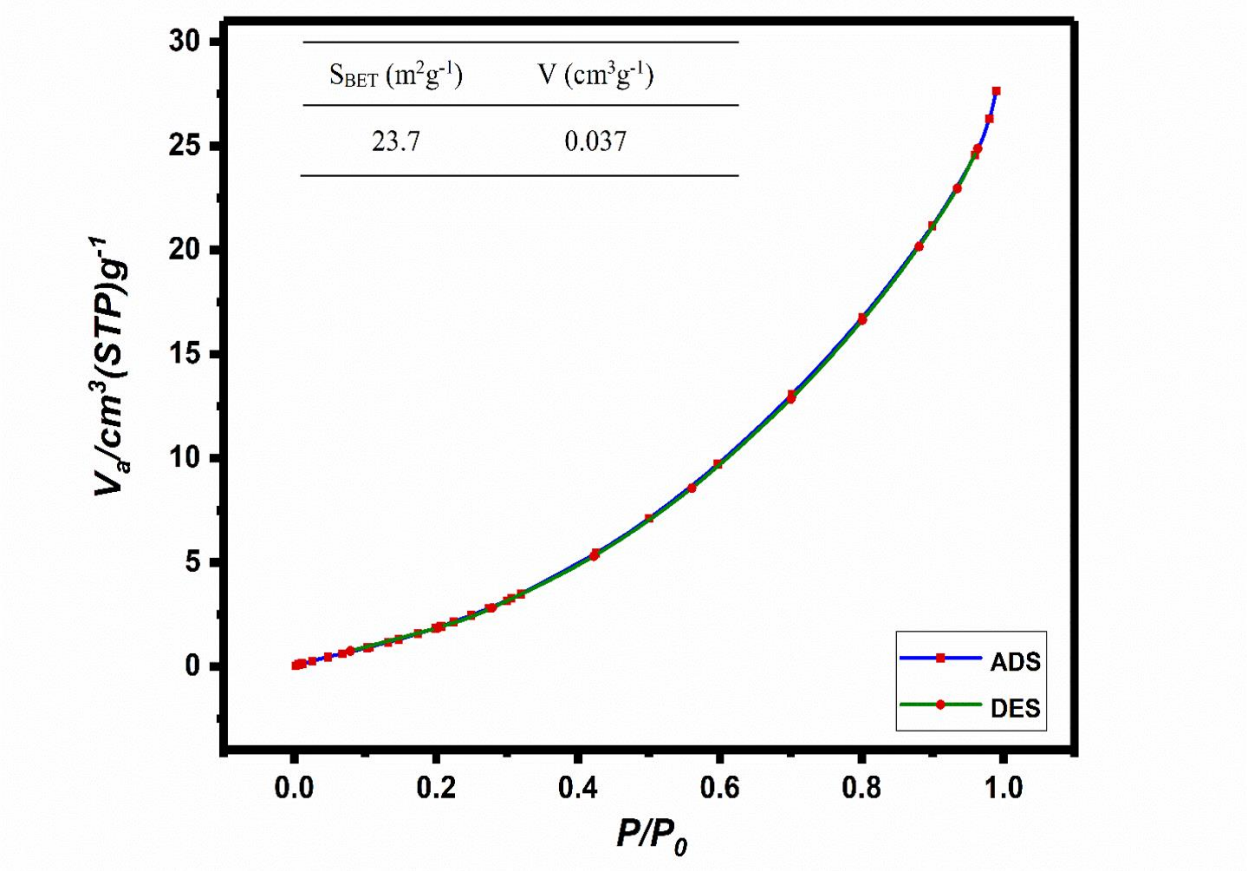
S_{BET} (m^2g^{-1}) V (cm^3g^{-1})
23.7 0.037
$V_a/cm^3(STP)g^{-1}$
P/P_0
ADS
DES
0
5
10
15
20
25
30
0.0
0.2
0.4
0.6
0.8
1.0

Figure 3

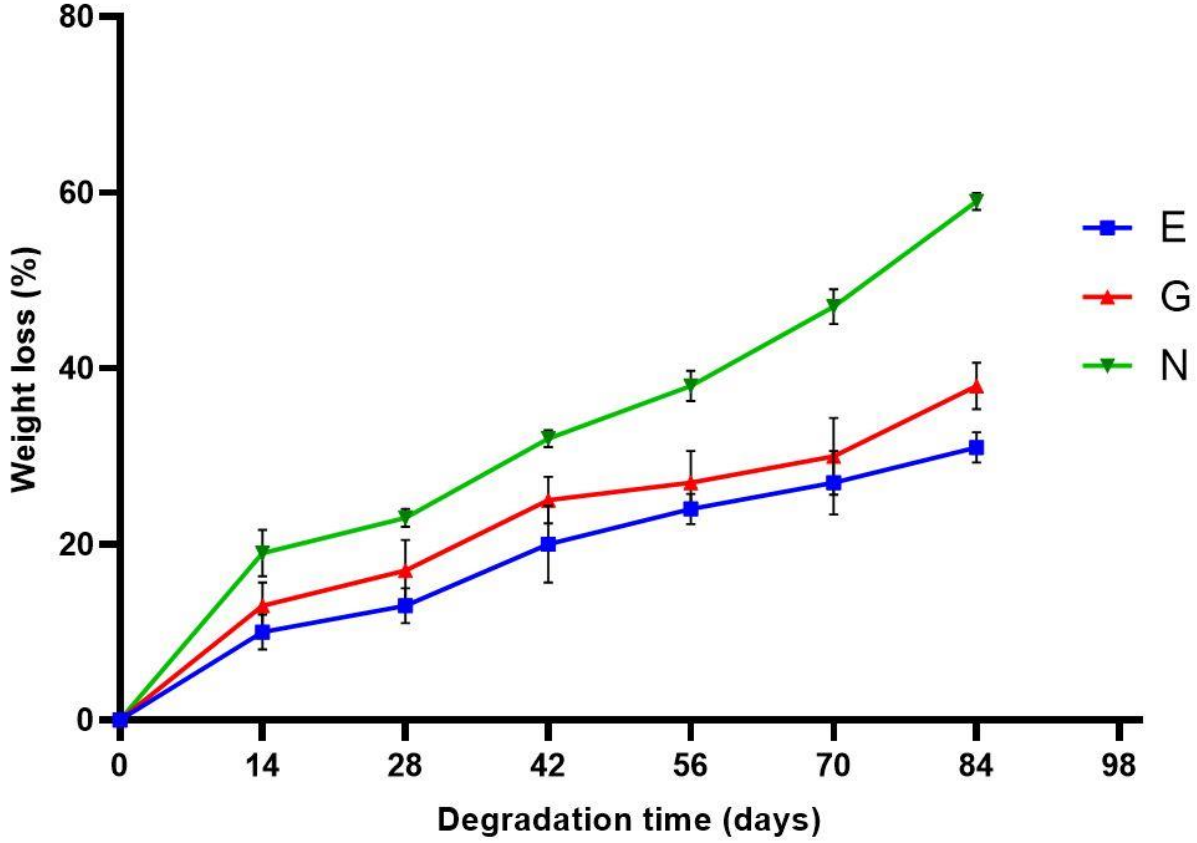
80
60
40
20
0
Weight loss (%)
0
14
28
42
56
70
84
98
Degradation time (days)
E
G
N

Figure 4

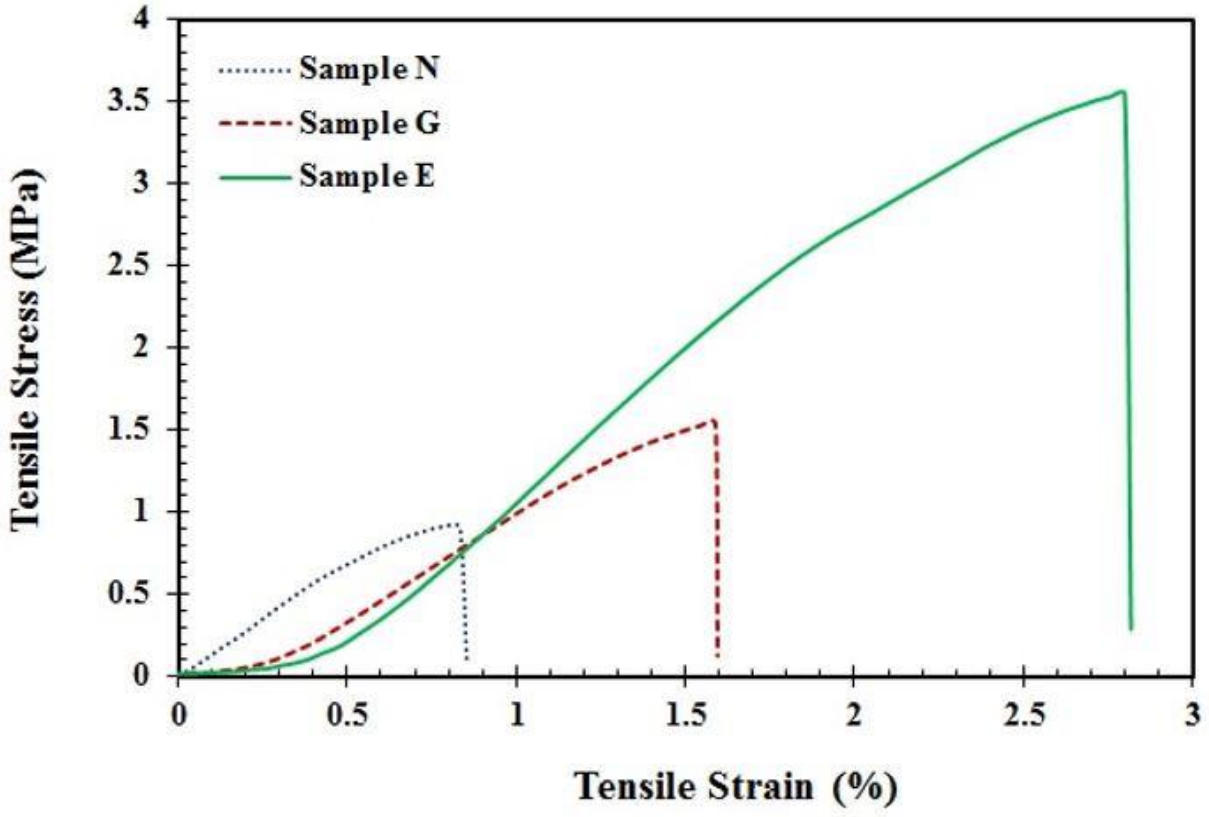
Sample N
Sample G
Sample E
Tensile Stress (MPa)
Tensile Strain (%)
0
0.5
1
1.5
2
2.5
3
3.5
4

Figure 5

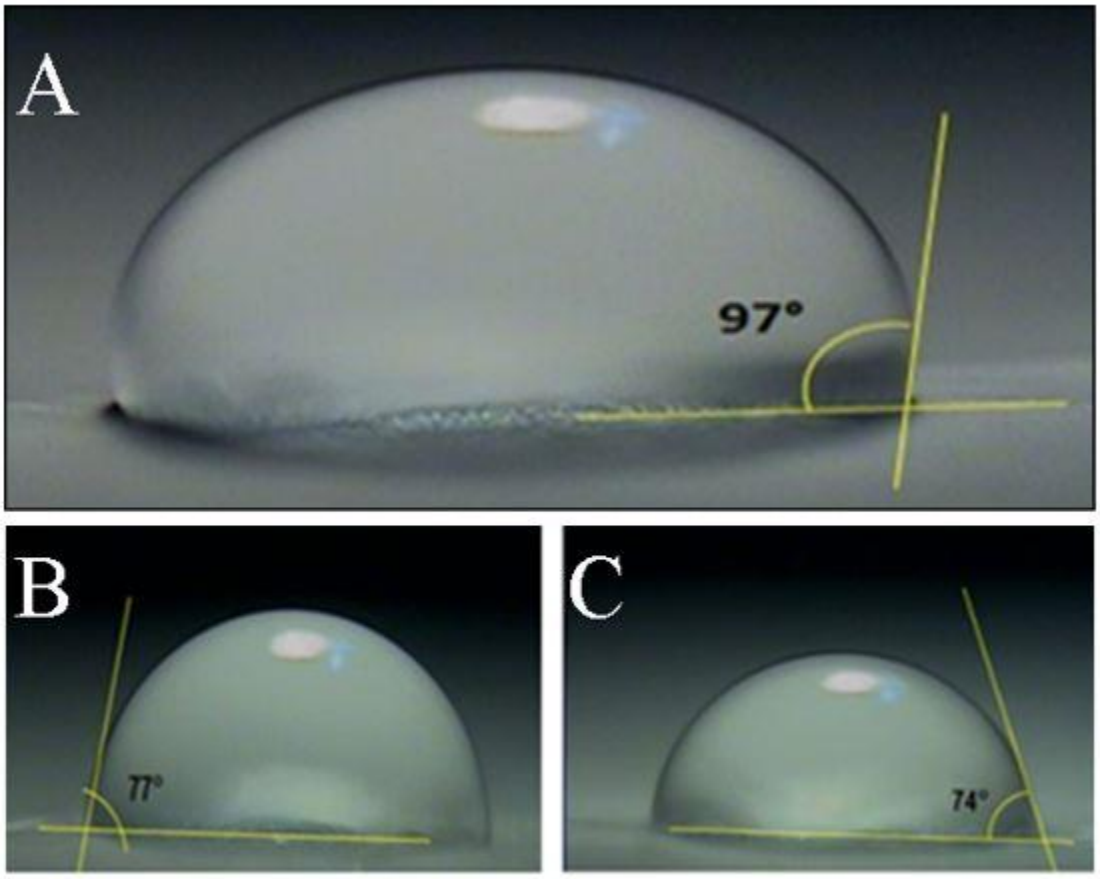

Figure 6

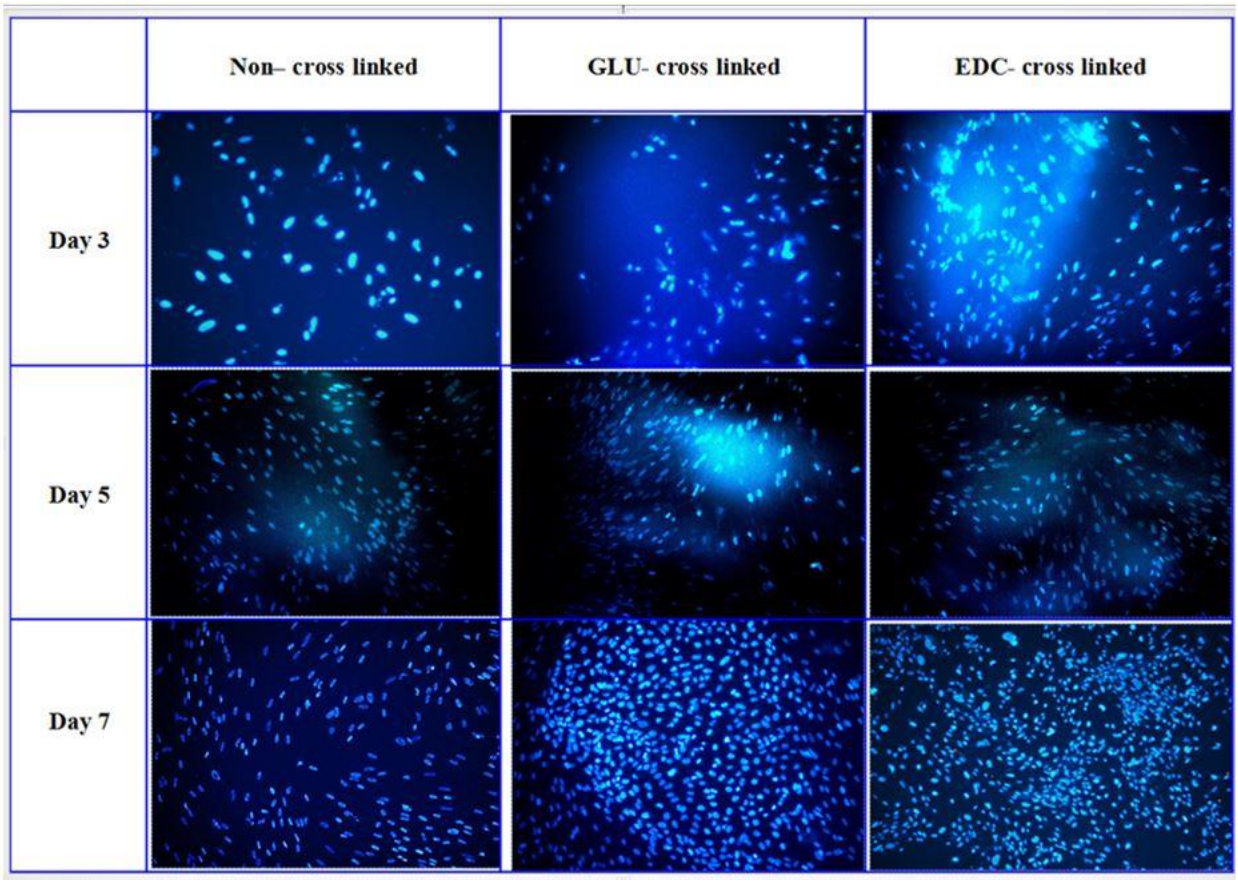
Non– cross linked
GLU- cross linked
EDC- cross linked
Day 3
Day 5
Day 7

Figure 7

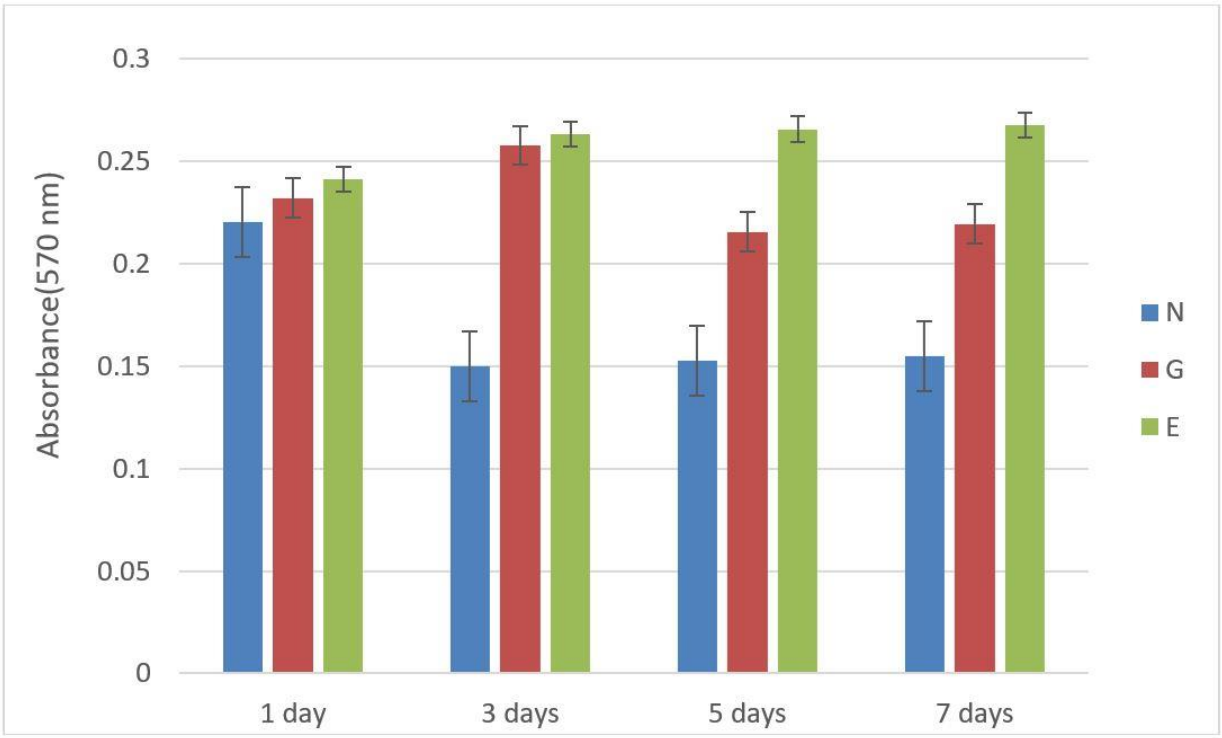

Figure 8

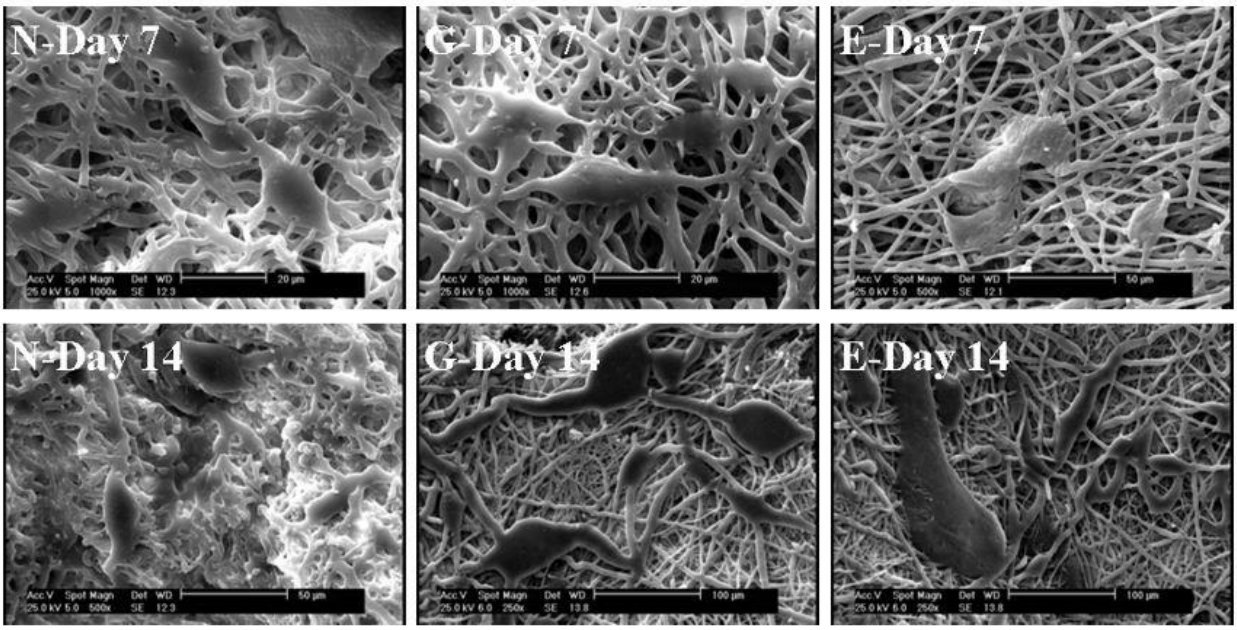

Table 1

Sample	Young'Modulus (Mpa)	Tensile Strength (Mpa)	Strain at Break (%)
N	1.3 ± 0.2	0.92 ± 0.1	0.8 ± 0.3
G	1.05 ±0.1	1.54 ± 0.3	1.5 ± 0.4
E	1.6 ± 0.3	3.5 ± 0.6	2.8 ± 0.7