(REFEREED RESEARCH)

FABRICATION OF ELECTROSPUN POLY (ETHYLENE TEREPHTHALATE) SCAFFOLDS: CHARACTERIZATION AND THEIR POTENTIAL ON CELL PROLIFERATION *IN VITRO*

ELEKTRO ÇEKİM YÖNTEMİ İLE POLİETİLEN TEREFTALAT DOKU İSKELESİ ÜRETİMİ: KARAKTERİZASYONU VE *IN VITRO* ORTAMDA HÜCRE ÇOĞALMASINDAKİ POTANSİYELİ

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ABSTRACT

Fiber diameter and fiber mat porosity play a key role on cell adhesion and proliferation. Therefore, in this study, poly(ethylene terephthalate) (PET) scaffolds consisting of fibers with different diameters and pore sizes were fabricated from solutions with different concentrations (10, 15 and 20% wt.) by electrospinning. Also, the effect of fiber mat thickness was investigated. The scaffolds were characterized in terms of surface and mechanical properties. The electrospun fibers had diameters ranging from 0.575 to 2.825 μm depending on the polymer concentration. Contact angle values showed that PET scaffolds had super hydrophobic structure. After characterization, fibroblast cells were cultured onto PET scaffolds and influence of different electrospinning parameters on cell proliferation was discussed. Fibroblast cells showed better proliferation on scaffolds consisting of smaller diameters. After evaluation of fibroblast cell proliferation on electrospun PET scaffolds, two different electrospun scaffolds were chosen: 1) scaffold with the smallest fiber diameter and 2) scaffold with better mechanical properties. Endothelial and keratinocyte cells were cultured on those two electrospun scaffolds. Cell adhesion and proliferation behavior of endothelial and keratinocyte cells on those scaffolds were investigated.

Keywords: Electrospinning, polyethylene terephthalate, scaffold, cell proliferation, fibroblast, endothelial, keratinocyte

ÖZET

Lif çapı ve yüzey gözenekliliği hücre tutunması ve çoğalmasında önemli bir rol oynamaktadır. Bu nedenle bu çalışmada, farklı lif çaplarına ve yüzey gözenekliliğine sahip polietilen tereftalat (PET) doku iskeleleri elektro çekim yöntemi ile farklı PET konsantrasyonuna sahip çözeltilerden (ağırlıkça %10,%15 ve %20) üretilmiştir. Ayrıca, üretilen yüzeylerin kalınlığının etkisi incelenmiştir. Doku iskelelerinin yüzeysel ve mekaniksel karakterizasyonu yapılmıştır. Elektro çekim yöntemi ile üretilen liflerin polimer konsantrasyonuna bağlı olarak 0.575 μm'den 2.825 μm'ye değişen aralıkta çaplara sahip olduğu görülmüştür. Temas açısı ölçümleri PET doku iskelelerinin süper hidrofobik yapıya sahip olduğunu göstermiştir. Karakterizasyon sonrası, PET doku iskeleleri üzerine fibroblast hücre ekimleri yapılmış ve farklı elektro çekim parametrelerinin hücre çoğalması üzerine etkisi araştırılmıştır. Fibroblast hücreleri küçük çaplara sahip liflerden oluşan yüzeyler üzerinde daha iyi çoğalmıştır. PET doku iskeleleri üzerine fibroblast hücre çoğalması davranışı incelendikten sonra, üretilmiş olan dokuz yüzeyden en küçük lif çapına sahip olan ve daha iyi mekanik özelliklere sahip olan iki yüzey seçilmiştir. Bu iki yüzey üzerine endotel ve keratinosit hücre ekimleri yapılmıştır. Endotel ve keratinosit hücrelerinin bu iki yüzey üzerindeki hücre tutunması ve çoğalması davranışları incelenmiştir.

Anahtar Kelimeler: Elektro çekim, polietilen tereftalat, doku iskelesi, hücre çoğalması, fibroblast, endotel, keratinosit

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1. INTRODUCTION

Electrospinning is a technique which uses electrostatic forces to produce sub-micron scaled fibers. In electrospinning process, a high voltage electrostatic force is applied to the polymer solution held in a syringe. Under the influence of the electrostatic field between the nozzle of a syringe and the collector, a jet of a polymer is ejected from the syringe and moves toward the collector. The solvent evaporates during the travel of the jet and thin dry fibers are deposited in the form of non-woven fabric on the metal collector. These electrospun fiber mats have potential applications especially in biomedical applications such as wound healing, tissue engineering and drug delivery because of their superior properties such as large surface area, high mechanical properties, small pore sizes and high porosity [1,2].

A tissue scaffold provides a temporary base for cells until the native extracellular matrix (ECM) is regenerated or repaired [3]. ECM has a nano-scaled structure. Therefore, a scaffold should meet several requirements such as biocompability, mechanical strength during the repair, appropriate pore size and porosity for cell attachment and growth. The scaffold should be produced from a biocompatible material and it should integrate with the host tissue without promoting or adverse any tissue reaction [4-7]. Porosity of the scaffold has crucial importance since it provides binding cites for cell attachment and also aids in nutrient transport [4-7]. Biodegrability is another factor for tissue scaffolds. Since there is no need for another surgery to remove the scaffold after the injured part is healed, biodegradable polymers are preferred. However, in some cases such as blood vessel, bone, skin and soft tissue repairs, biodegradability is not necessarily required. In such cases, non-biodegradable, biocompatible polymers with good mechanical properties are preferred [4-7].

Poly(ethylene terephthalate) (PET) is a biocompatible, non-biodegradable, non-toxic,non-cancerogenous, non-allergenic, biostable linear polymer with good mechanical and thermal properties and widely used in medical applications such as surgery sutures, medical gowns, hospital sheets, vessel grafts and ligaments. In the present work, PET was chosen due to such desirable properties [8].

In humans and other mammals there are more than 200 different types of cells, all of which are structurally and biochemically specialized to carry out a particular function [9]. Each cell shows different proliferation and growth behavior. Therefore, in cell culture studies, cell line type should be selected according to where the cells will function. Fibroblast cells can be chosen for soft tissue or connective tissues, while endothelial cell lines for blood cells and keratinocyte cell lines for skin [9].

The aim of this study is to fabricate electrospun PET scaffolds and understand effect of scaffolds characteristics (fiber diameter, pore size, porosity) on cell proliferation *in vitro*. Therefore, PET scaffolds were produced by electrospinning with different solution concentrations and system parameters (flow rate and collector speed). Distance and voltage kept constant since they are dependent variables. The scaffolds were characterized in terms of surface and mechanical properties. Fibroblast cell culture studies were performed on scaffolds and effect of scaffold characteristics on cell adhesion and proliferation was discussed. After evaluation of fibroblast cell proliferation on the samples, two of the scaffolds were selected and endothelial and keratinocyte cells were cultured on them in order to observe proliferation behavior of different cell types.

2. MATERIALS AND METHODS

2.1. MATERIALS

In this study commercially available textile grade PET polymer was used to produce electrospun PET scaffolds. The viscosity of the chips was 0.645 P. The chemicals dichloromethane (DCM) and trifluoroacetic acid (TFA) were used as solvents to dissolve the PET polymer. All chemicals were used as received without any further purification. All chemicals were purchased from Bursa Teknik Kimya Tic. ve Pazarlama Ltd Şti. (Bursa, Turkey).

Fibroblast (NHDF-Ad-Adult Human Dermal fibroblasts-Lonza), Endothelial (HMVEC- dBlAd-Adult Human Dermal Blood Microvascular Endothelial Cells) and Keratinocyte (NHEK-Adult Normal Human Epidermal Keratinocytes-Lonza) cell lines were purchased from Aka Biotechnology Ltd. Şti. (İstanbul, Turkey)

2.2 METHODS

2.2.1 FABRICATION OF ELECTROSPUN PET SCAFFOLDS

PET scaffolds were electrospun from three solutions with different concentrations (10, 15 and 20% wt.). PET chips were dissolved in a mixture of trifluoroacetic acid: dichloromethane (TFA:DCM) (50:50 w/w). In order to obtain a homogenous mixture, the solutions were kept at room temperature on the magnetic stirring plate for 4 h. Intrinsic viscosities of all solutions were measured by a Brookfield (RDV-II + Pro Extra) viscometer at 100 rpm. Temperatures of the solutions were at 19°C, approximately. Electrospinning was performed with an electrospinning unit (NanoSpinner24, Inovenso) with different parameters (Table 1). Distance and voltage kept constant at 10 cm and 20 kV for all samples since they are dependent variables. All experiments were carried out in air at room conditions. The thickness of the produced electrospun PET mats were measured by an Electronic Digital Micrometer.

Solution preparation, viscosity measurements of the solutions, fabrication of the scaffolds and mat thickness measurements were performed in Textile Engineering Department, Uludag University (Bursa, Turkey).

Table 1. Electrospinning parameters of PET mats

Sample Code	PET Concentration (%)	Flow Rate (ml/hr)	Collector Speed (rpm)
P1	10	1	250
P2	10	1.5	250
P3	10	1	350
P4	15	1	250
P5	15	1	350
P6	15	1	500
P7	15 (thinner mat)	1	250
P8	15 (thicker mat)	1	250
P9	20 PET	1	250

2.2.2. CHARACTERIZATION OF ELECTROSPUN PET SCAFFOLDS

SEM Studies: The surface morphologies of the PET scaffolds were evaluated by Scanning Electron Microscopy (SEM) (Carl Zeiss Evo 40) at 20 kV. Fiber orientation in the mat, fiber diameter changes, fiber evenness, fiber surface roughness and pore sizes of the mats were evaluated. The surfaces were coated with a thin conducted gold layer prior to SEM observations. Fiber thickness measurements were carried out during SEM analysis on the image. 20 measurements were performed for each sample and average values were recorded.

Cell adhesion and proliferation were also investigated by SEM studies. Cell cultured nanofiber mats were dried by a critical point dryer before SEM analysis.

Pore Size and Porosity: The mean effective pore diameter (pore size) and porosity of the electrospun mats were calculated using the following equations [10,11]:

$$\varepsilon = \left(1 - \frac{\rho e}{\rho f}\right) x 100\% \quad (1)$$

Where ε is the porosity of the non-woven fabric, ρ_e and ρ_f are the densities of the electrospun fiber mat and fiber, respectively. The bulk density of the PET (ρ_f) is taken as 1.3 g cm^{-3} [11].

And the mean effective pore diameter (pore size) of electrospun fiber is given by:

$$D = \frac{\pi d}{4(1 - \varepsilon)} \quad (2)$$

Where D is the mean effective pore diameter, d is the mean fiber diameter and ε is the mean porosity of the sample.

Contact Angle Measurements: The contact angle of the electrospun PET scaffolds were measured using KSV-The Modular CAM 200 contact angle measurement system. A distilled water drop was dispersed on each sample using a micropipette; the image of each drop was captured by the camera connected with a computer based image capture system. The images were captured as quickly as possible after water droplet was placed onto the sample surface, and photographed in less than 1 s.

Tensile Tests: Tensile tests were carried out on a 4301 Instron tensile tester with a gauge length of 20 mm and a crosshead speed of 20 mm/min [12,13]. The samples were cut rectangular with dimensions of 5x50 mm.

SEM analyses were performed in Physics Department, Uludag University and contact angle measurements and tensile tests were performed in Textile Engineering Department, Uludag University (Bursa, Turkey).

2.2.3. STERILIZATION AND CELL CULTURE STUDIES

Before cell culture studies, the samples should be sterilized. Among many sterilization methods, UV is preferable since it does not give any damage to the electrospun fiber mat [8, 14]. Samples were washed with ethylene alcohol and phosphate buffered saline (PBS) solution for 3 times, respectively. Afterwards, samples were sterilized in the cabinet (Thermo, Hera guard, model HPH) under UV light for 1 hour. The procedure was repeated for both sides of the samples. All the sterilizations were carried out according to the standard procedures used in the sterilization unit of the Medical Faculty, Uludag University.

Fibroblast, endothelial and keratinocyte cell lines were cultured in 25 cm^2 flasks at 37°C in an incubator with 5% humidified CO_2. The cultures were replenished with medium (LONZA) at 37°C every 3 days. When primary cells became near confluent, they were detached from the flasks with 0.05% trypsin-EDTA solution (Invitrogen) for 7 min at 37°C for expanding cells. After a series of passaging procedure, the cells were seeded separately on the PET scaffolds held on the bottom of a 6-well tissue culture plate in an appropriate culture medium.

The growth and proliferation of a cell needs a period of time. To determine the required time for a cell to proliferate, a reference group is needed. Therefore, another group of fibroblast, endothelial and keratinocyte cells were cultured onto the flasks without a fiber mat as a reference group simultaneously and their proliferation was followed. After the reference group covered the flask surface, electrospun scaffolds were removed from the culture medium and prepared for SEM analyses.

Cell culture studies were performed in Prof. Dr. Sermin Paker Cell and Embryo Culture Laboratory in Histology and Embryology Department of Medical Faculty, Uludag University (Bursa, Turkey).

3. RESULTS AND DISCUSSION

3.1. CHARACTERIZATION OF ELECTROSPUN PET SCAFFOLDS

For cell attachment and proliferation, electrospun fiber mat characteristics such as surface topography, mat thickness, fiber diameter, uniform diameter along the fiber length and

pore size are important. In this study scaffolds with different fiber diameter, pore size and mat thickness were fabricated by changing the polymer concentration, flow rate and collector speed (Table 1).

P1, P2 and P3 samples were fabricated from 10% wt. PET solutions. Effect of flow rate effect and collector speed was investigated. When P1 and P2 samples were compared, P2 was fabricated from higher flow rates. Beads were seen in both surfaces. However, thicker fibers were produced in P2 due to higher flow rate (Table 2). P3 scaffold was produced from higher collector speed. With the increasing collector speed, electrospun mats with thinner fibers and less beads were fabricated (Figure 1).

In order to understand the fiber diameter distribution within the fiber mat, frequency distribution graphs were given for each sample. It can be seen that P1 sample have fibers mostly with diameters in the range of 700-1000 nm whereas P2 sample have in the range of 300-500 nm. P3 samples have fibers mostly with diameters ranging from 200-500nm and 500-1000 nm.

P4-P6 samples were fabricated from 15% wt. PET solutions with different collector speeds. Changing collector speed resulted in smaller fiber diameters (Table 2, Figure 2). When the speed of the collector was 500 rpm (P6), the fiber formation was interrupted. Therefore, the optimum collector speed was determined as 350 rpm.

In order to understand the effect of fiber mat thickness on cell proliferation, thinner and thicker electrospun fiber mats were fabricated from 15% wt. PET solutions, denoted as P7 and P8 (Figure 3). The fibers had similar diameter values as in P4-P6. P9 was fabricated from 20% wt. PET solutions. With the increasing polymer concentration, thicker fibers were produced. The sample had uniform fibers with the highest diameter values (Table 2).

Effect of polymer concentration on fiber mat characteristics was investigated and three electrospun fiber mats were produced from 10% wt., 15%wt., and 20% wt. PET solutions, denoted as P1, P4 and P9.

In electrospinning, polymer concentration directly affects the viscosity of the solution which leads to different mat structures [4,5]. Viscosity of the PET solutions were as 38.40, 115.20 and 278.40 cP for 10% wt., 15%wt., and 20% wt. PET solutions, respectively. Therefore, fibers with larger diameters and fewer defects were formed with the increasing polymer content (Table 2). Moreover, the fiber cross-section became more uniform, larger electrospun fibers were produced and thicker mat was obtained with the increasing polymer concentration (Figure 1a, 2a, 3c).

The free surface energy of the fibers is one of the main characteristics because it determines the potential level of interactions that the fibers are able to have with other fibers. It is necessary to know the surface energy to understand many surface characteristics, such as wettability, adhesion, friction, and absorption. Contact angle is associated with the free surface energy. Higher free surface energy results in lower contact angle. Conventional PET fibers exhibit a contact angle around 70–75°C, which is associated with low free surface energy [15].

Electrospun PET fiber mats showed super hydrophobic surface characteristics. The decrease in fiber diameter resulted in rough surface which leads to lower contact angles (Table 2).

Electrospun fiber mats are favorable for tissue engineering applications due to their superior properties such as large surface area, high mechanical properties, small pore sizes and high porosity [1,2].

In tissue engineering applications one of the most important parameter is the pore properties (such as porosity, pore size and volume) of the scaffold since it provides binding cites for cell attachment and also aids in nutrient transport. PET electrospun scaffolds showed ≈80% porosity which is compatible with previous studies with PET electrospun fiber mats [11]. Also, PET scaffolds featured pore sizes with a few micrometers. In literature, it was reported that fibroblasts cells have diameters about 10-15 μm. This indicates pore sizes with a few microns are adequate for cell migration (Table 2) [16].

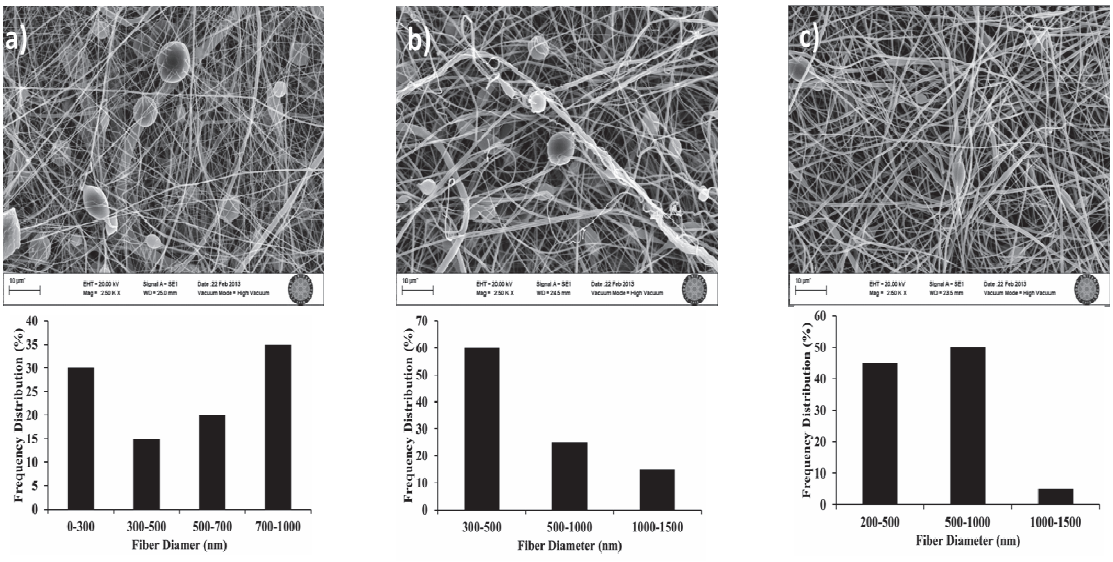

Figure 1. SEM images and fiber diameter distribution of a) P1, b)P2, c)P3 respectively.

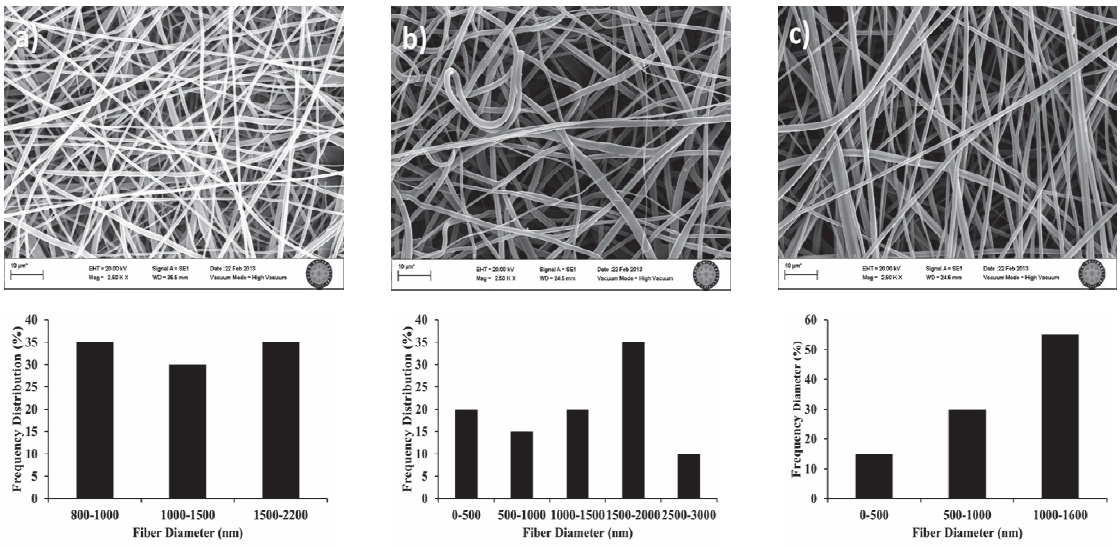

Figure 2. SEM images and fiber diameter distribution of a)P4, b)P5, c)P6, respectively.

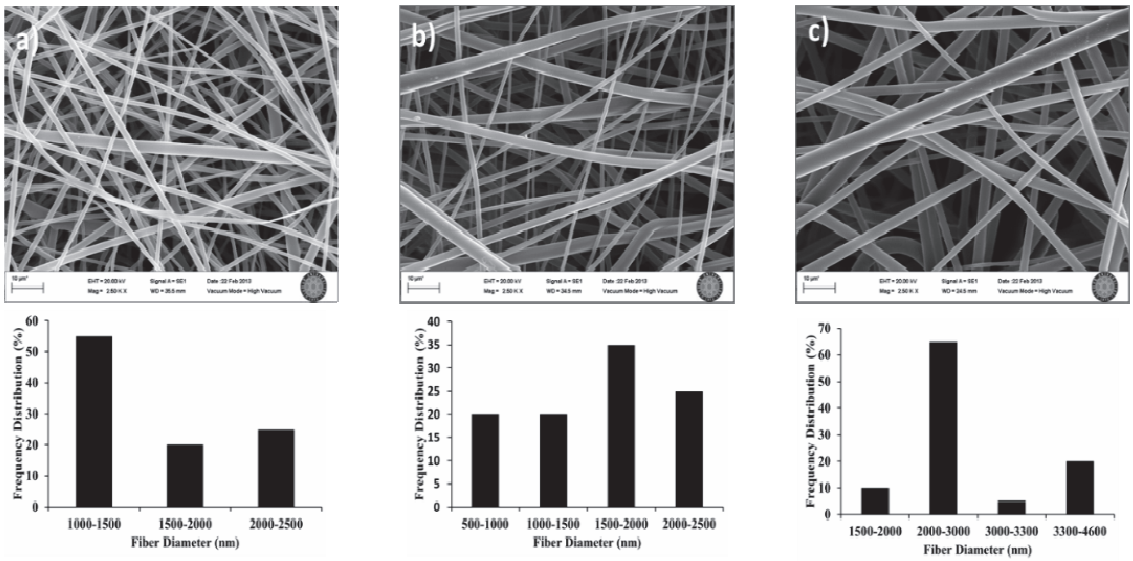

Figure 3. SEM images and fiber diameter distribution of a) P7, b) P8, c) P9, respectively.

Table 2. Properties of the electrospun PET mats

Sample Code	Diameter (μm)	Mat Thickness (mm)	Contact Angle (°)	Pore size (μm)	Porosity (%)
P1	0.587±0.276	0.121±0.00586	116.37±3.40	2,42	80,96
P2	0.600±0.316	0.298±0.00893	119.78±1.20	1,79	73,72
P3	0.575±0.252	0.352±0.00508	115.87±2.93	2,79	83,83
P4	1.375±0.445	0.281±0.00464	128.86±3.80	7,58	85,75
P5	1.368±0.665	0.365±0.00702	128.80±7.60	9,77	89,01
P6	0.900±0.357	0.349±0.00606	128.98±4.25	4,00	82,35
P7	1.525±0.492	0.149±0.00385	131.85±1.81	3,65	67,17
P8	1.575±0.487	0.656±0.00313	130.71±5.70	7,59	83,71
P9	2.825±0.791	0.386±0.00404	132.94±5.66	10,43	78,74

Mechanical properties are not the most effective parameter on cell growth and proliferation. Since the scaffold should maintain its mechanical properties during healing process, the tensile tests were performed on electrospun PET fiber mats.

When P4, P5 and P6 are considered, the increase of Young modulus can be mainly attributed to the increase of molecular orientation due to the increase in the speed of the rotating drum. However, in mechanical properties of electrospun fiber mats, uniform fiber diameter and thickness are also important as well as molecular orientation. The high modulus and low elongation break values of P9 can be explained by the uniform and thicker fiber diameter and narrow fiber diameter distribution [17].

P9 samples fabricated from 20% wt. PET solutions gave the highest modulus and the lowest elongation at break values (Table 3).

Table 3. Tensile properties of the electrospun fiber mats

Sample Code	Elongation at Break (%)	Young Modulus (MPa)
P1	70.57±4.24	39.87±7.91
P2	97.26±5.66	51.78±8.77
P3	101.38±9.27	47.39±6.48
P4	90.08±4.43	174.60±7.82
P5	111.90±10.74	199.10±13.23
P6	91.19±8.74	265.72±17.55
P7	100.50±15.27	169.50±9.28
P8	112.70±15.85	171.20±11.01
P9	64.24±10.33	332.20±23.39

3.2 CELL CULTURE STUDIES

After the samples were sterilized by UV method, cell culture studies were performed on the electrospun PET mats. Fibroblast cells did not show any proliferation on the samples produced from 20% wt. PET solutions which had the highest diameter and pore size (Figure 4i).

Fibroblast cells showed good proliferation mostly on the samples produced from 10% wt. PET solutions (4a-c). This is associated with lower fiber diameter, pore size and high porosity. Especially on P3 which has the lowest fiber diameter, fibroblast cells covered the surface of the mat. Also, the decrease in fiber diameter leads to rough surfaces. The roughness can act as the binding sites where cells can attach [16].

Due to the increasing fiber diameter and pore size, fibroblast cell proliferation wasn't as good as on the samples produced from 15% wt. PET solutions compared to the samples produced from 10% wt. PET solutions (4d-h). On P7 and P8 samples, fibroblast cells showed similar cell adhesion and proliferation as in the samples produced from 15%wt. PET solutions (4g-h). This result shows that fiber mat thickness doesn't have significant importance on cell adhesion and proliferation.

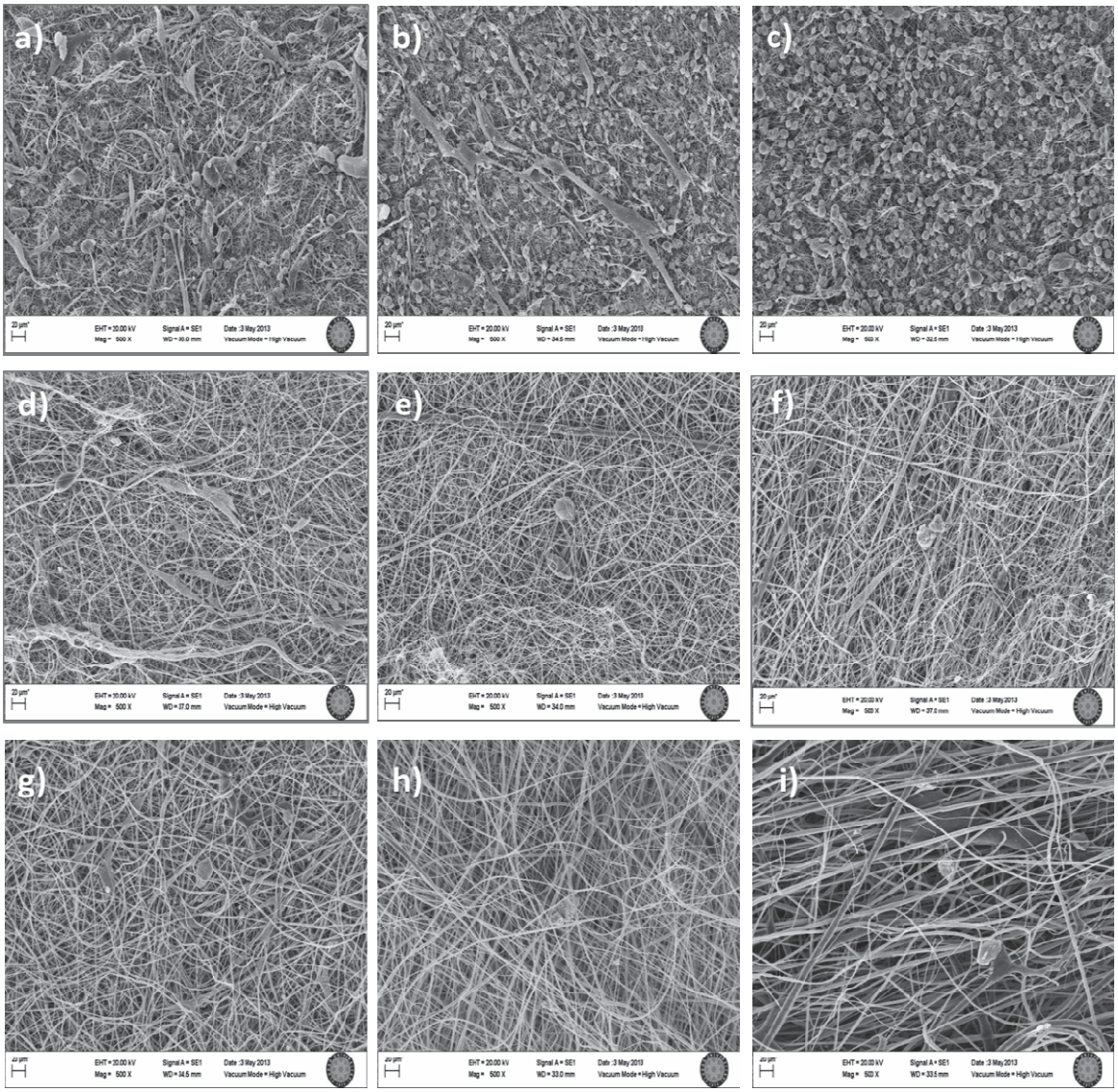

Figure 4. Fibroblast cell proliferation on electrospun PET scaffolds; a)P1, b)P2, c)P3, d)P4, e)P5, f)P6, g)P7, h)P, i)P9, respectively.

Fibroblast cell culture studies showed that smaller fiber diameters and pore sizes favor cell growth and proliferation. In order to support this hypothesis, endothelial and keratinocyte cells were cultured on P3 which had the best fibroblast cell proliferation. The scaffold should maintain its mechanical properties during healing. Therefore, endothelial and keratinocyte cells were also cultured on P5 which has good mechanical properties.

Endothelial cell proliferation followed the same trend as fibroblast cells. They showed good cell adhesion on P3. The cells proliferated well and covered the scaffold surface. The cells did not show good adhesion and proliferation on P5 surfaces, which has larger diameters (Figure 5).

Although keratinocyte cells showed more proliferation on P3 compared to P5, they did not covered the surfaces (Figure 6). This can be explained by their characteristics. Keratinocyte cells are already difficult cells to isolate and grow. They also have a limited lifespan [18,19]. This shows cell characteristics also play an important role on cell proliferation.

4. CONCLUSION

PET fiber mats from different polymer concentrations, flow rate and collector speed were successfully produced by electrospinning to be used as tissue scaffolds. Also, the effect of fiber mat thickness on cell proliferation was investigated.

All electrospun PET fiber mats showed super hydrophobic structure. The decrease in fiber diameter resulted in rough surface which leads to higher contact angles. Surfaces fabricated from lower concentration PET solutions gave lower pore sizes.

P9 sample fabricated from 20% wt. PET solution gave the highest modulus and the lowest elongation at break values, as expected.

Fibroblast cells covered the surface of P3. This indicates that lower fiber diameter and small pore size play a key role on cell adhesion and proliferation. With the increasing fiber diameter cell proliferation on the mats were decreased. On P7 and P8 samples, fibroblast cells showed similar cell adhesion and proliferation as in the samples produced from 15%wt. PET solutions. It can be concluded that mat thickness did not have crucial importance on cell proliferation.

Endothelial and keratinocyte cells were cultured on two different surfaces: scaffold consisting of smaller diameters and scaffold with good mechanical properties. Endothelial cell proliferation followed the same trend as fibroblast cells and proliferated well. They did not proliferate on the scaffolds with larger diameters. Keratinocyte cells, which have limited life span and hard to culture, showed more proliferation on the scaffold with smaller diameters compared to the scaffold with larger diameters. However, the cells did not cover the surfaces of both scaffolds. This can be associated with their cell characteristics.

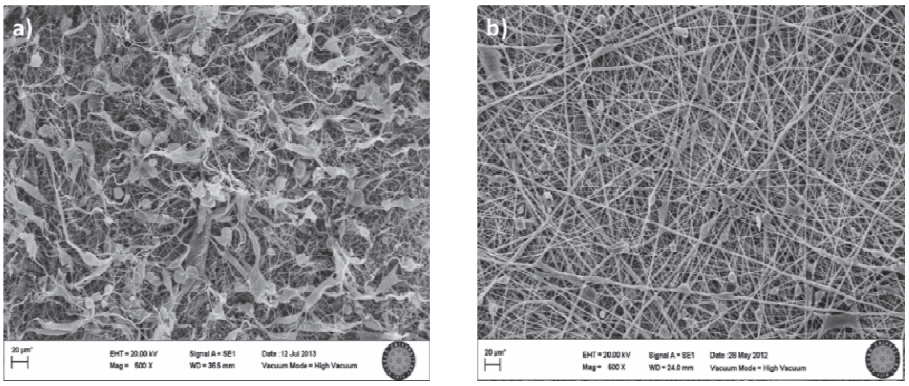

Figure 5. Endothelial cell proliferation on electrospun PET scaffolds, a)P3, b)P5, respectively.

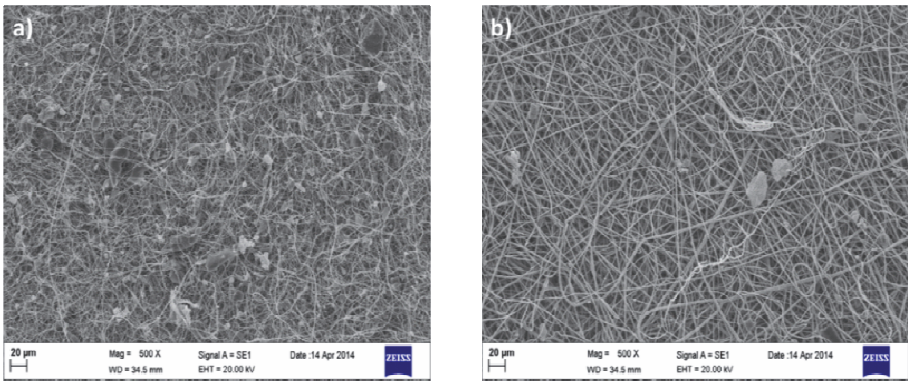

Figure 6. Keratinocyte cell proliferation on electrospun PET scaffolds, a)P3, b)P5, respectively.

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