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Electrospun Poly(lactic acid-co-glycolic acid) Scaffolds for Skin Tissue Engineering

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Abstract

Electrospun fiber matrices composed of scaffolds of varying fiber diameters were investigated for potential application of severe skin loss. Few systematic studies have been performed to examine the effect of varying fiber diameter electrospun fiber matrices for skin regeneration. The present study reports the fabrication of poly[lactic acid-co-glycolic acid] (PLAGA) matrices with fiber diameters of 150–225, 200–300, 250–467, 500–900, 600–1200, 2500–3000 and 3250–6000 nm via electrospinning. All fiber matrices found to have a tensile modulus from 39.23 ± 8.15 to 79.21 ± 13.71 MPa which falls in the range for normal human skin. Further, the porous fiber matrices have porosity between 38–60 % and average pore diameters between 10–14 μm . We evaluated the efficacy of these biodegradable fiber matrices as skin substitutes by seeding them with human skin fibroblasts (hSF). Human skin fibroblasts acquired a well spread morphology and showed significant progressive growth on fiber matrices in the 350–1100 nm diameter range. Collagen type III gene expression was significantly up-regulated in hSF seeded on matrices with fiber diameters in the range of 350–1100 nm. Based on the need, the proposed fiber skin substitutes can be successfully fabricated and optimized for skin fibroblast attachment and growth.

Keywords

Electrospinning; Skin Grafts; PLAGA; Fiber matrices; Tissue Engineering; Human skin fibroblast; Collagen

Introduction

Successful wound healing is a complex phenomenon involving interactions between epidermal and dermal cells, the extracellular matrix (ECM), and angiogenesis; all of which are regulated by an array of cytokines and growth factors [1]. Traditionally, autografts and allografts have been used to treat burns or other full thickness skin defects. Autografts have a higher success rate but are limited in supply and may cause donor site morbidity. Allografts though abundant,

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have always presented risk for disease transmission and immunological rejection. Tissue engineering has emerged as a promising alternative to treat skin injuries and/or defects. Such an approach involves scaffolds, cells and biological cues alone or in combination [2].

Wounds with large amounts of skin loss require immediate coverage with a dressing primarily to protect the wound. An ideal dressing would mimic the functions of native skin; protecting the injury from loss of fluid and proteins, enabling the removal of exudates, inhibiting exogenous microorganism invasion, and improving aesthetic appearance of the wound site [3,4]. Bandages with the desired physiochemical properties have been fabricated into various forms such as films, microfiber meshes, and sponges using synthetic polymers [5,6]. Critical determinants leading to favorable wound healing outcomes are largely based on the choice of polymer, fabrication methodology, cell phenotype and surface topography. Nanotopographical features such as pores, ridges, groves, fibers, nodes or a combination of these have been reported to influence cell behavior [7–11].

In that direction, scaffolds composed of nano or micro diameter fibers mimic the structure and morphology of the ECM components in the skin [6,12–14]. Electrospinning has emerged as an efficient technique to produce nano and micro-diameter fibers by manipulating polymer concentrations and various process parameters [6,12–14]. The electrospun scaffolds have high surface area-to-volume ratio and thus provided more surface area for cell attachment as compared to 3-dimensional (3-D) scaffolds made using other techniques. The high surface area prevented fluid accumulation and more facile oxygen permeation [14–16].

Post-surgical adhesion is a major challenge that affects wound healing and occurs with the use of either conventional bandages or barrier devices [17]. Such adhesions would require a second surgical procedure that might complicate the wound healing response, formed potential scar tissue, increase the risk of bacterial infection, and would be time consuming and costly. Attempts made to prevent post-surgical adhesions using irrigation, various fibrinolytic agents, and physical barriers comprised of polymeric gels have been unsuccessful [18,19]. A successful study towards the prevention of post-surgical adhesion in a rat model utilized PLAGA electrospun nonwoven bioabsorbable nanofiber matrices as bandages and showed excellent anti-adhesion effect, and prevented complete cecal adhesions [20]. Studies involving fibroblasts have quantified a more desired cell behavior on biomaterials that are composed of nano-scale architecture as compared to micro-scale features [10,15,21,22]. However, the specific characteristics of these nonwoven nanofiber matrices that would modulate a more favorable human skin fibroblast response have not being explored. This article explores hSF response to nonwoven electrospun PLAGA fiber matrices of varying fiber diameters to develop a suitable surface wound healing medical device. This comprehensive study evaluated proliferation and gene expression of human skin fibroblasts (hSF) on nonwoven PLAGA scaffolds of varying fiber diameters.

Materials and methods

Materials

Poly[(50% lactic acid)(50% glycolic acid)] (PLAGA), molecular weight $M_w = 71,000$ (Lakeshore Biomaterials Inc., Birmingham, AL), tetrahydrofuran (THF) and dimethylformamide (DMF) (Fisher Scientific, Atlanta, GA) were used for these studies. CellTiter 96® AQueous one solution was purchased from Promega (Madison, WI). Live/dead cell viability kit was purchased from Molecular Probes (L-3224). Human skin fibroblasts (hSF) (ATCC, Manassas, VA CRL-2072, CCD-1059SK) lot 3296816, and Eagles Minimum Essential Medium (EMEM) were obtained from ATCC. Fetal bovine serum (FBS), antibiotics (Penicillin, Streptomycin P/S) and trypsin–EDTA, were purchased from Sigma (St. Louis, MO).

Fiber matrix fabrication

Nonwoven fiber matrices were fabricated using a conventional electrospinning setup reported earlier [5,6,13] with a slight modification to the existing system [23]. The apparatus consists of a 10 mL glass syringe fitted with a 20 gauge blunt end needle and a grounded electrode covered with an aluminum foil sheet. PLAGA of molecular weight 71,000 was dissolved in an organic solvent mixture of THF:DMF (3:1 ratio) at varying concentrations. Polymer solution flow was adjusted using a programmable syringe pump (Genie, Kent Scientific Corporation USA) to a flow rate of 2mL/h. A Gamma High Voltage Supply ES40P-20W (0–40 kV, 20 W, Gamma High Voltage Research) with a low current output was used to maintain a potential gradient of 1kV/cm. A circular disc fitted on the needle connected to a positive lead of the power supply acts as circular electrode [23]. Such an arrangement helps to focus the polymer jet at the desired location on the target and avoids loss of polymer. Electrospinning was carried out at ambient temperature and pressure. The spun fiber matrices were dried under vacuum at room temperature for 24 h.

Scanning Electron Microscopy (SEM)

The morphologies of the nonwoven fiber matrices were characterized by SEM. The polymer coated surfaces were sputter coated with gold using a Hummer V sputtering system (Technics Inc., Baltimore, MD) before viewing with SEM. The samples were viewed using JSM 6400 scanning electron microscope (JEOL, Boston, MA, USA) operated at an accelerating voltage of 20 kV at various magnifications. The fiber diameters were determined by (Image J, NIH) measuring the diameters of randomly selected fibers at different locations on the sample (n=3). In each location 100 different fibers were selected for measurement.

Cell Culture

Human skin fibroblasts were plated in tissue culture flasks (125 cm²) and cultured in EMEM supplemented with 10% FBS and 1% P/S. The media was replaced every other day, and culture was maintained in a tissue culture incubator at 37°C and 5% carbon dioxide. Passage 6 cells were used for cell seeding.

Nonwoven fiber matrices were cut into circular discs using cork borer no. 10 with an area of approximately 2.27 cm² and a thickness of 0.38–0.42 mm. All matrices were soaked in 70% ethanol for 20 min, and then dried and sterilized under UV light for 1 h on each side. Scaffolds were placed in 24 well plates and washed by soaking completely in serum supplemented DMEM for 15 min to remove traces of alcohol and provide hydrophilic surfaces for optimal cell adhesion. Cell suspension was pipetted directly onto the scaffolds with an initial seeding density of 50,000 cells/scaffold and incubated for 1 h. Following 1 h incubation, the 1.8 mL of growth media was added to the samples and then changed completely every other day.

Cell Proliferation Assay

The cell proliferation on the surface of the nonwoven fiber matrices was determined at time points of 1, 3, 7, 14 and 28 days. Cell proliferation was measured using MTS assay (CellTiter 96® AQueous one solution cell Promega Corp., Madison, WI). The metabolically active cells react with the tetrazolium salt in the MTS reagent to produce a formazan dye that can be observed at λ_{max} 490 nm. At each time point cellular constructs were washed twice with PBS to remove non-adherent cells and then transferred to a new 24 well plate. These constructs were incubated with 200 μ L of MTS reagent with 1mL of serum free medium for 2h. Aliquots were taken and their absorbance was read on UV-Spectrophotometer (Shimadzu, Japan) at λ_{max} 490 nm. The absorbance of six known cell numbers 10,000, 30,000, 50,000, 75,000, 100,000, and 150,000, were used to construct a standard curve to convert absorbance readings to cell numbers.

Live/Dead cell Viability

Viability of hSF on nonwoven fiber matrices were imaged with a live/dead cell viability kit (Molecular Probes, L-3224). In brief, calcein AM enters live cells and reacts with intracellular esterase to produce a bright green fluorescence, while ethidium homodimer-1 enters only dead cells with damaged membranes and produces a bright red fluorescence upon binding to nucleic acids. Fiber matrices were imaged on 7, 14 and 28 days using a BioRad Radiance 2100 Multiphoton/Laser Scanning Confocal Microscope (LSCM) and a Nikon Eclipse E600 Fluorescent Microscope at different magnifications.

Cell Behavior on Fiber Matrices

Cellular constructs were harvested at 1, 4 and 12 h to know the initial hSF interaction with nonwoven fiber matrices of different diameters. Cellular constructs were harvested at later time points of 1, 3, 7, 14, and 28 days to quantify the cellular response and proliferation on these fiber matrices. At each time point, scaffolds were washed twice with PBS to remove non-adherent cells and fixed with 1% glutaraldehyde at 4°C for an hour, followed by fixation with 3% glutaraldehyde overnight at 4°C. Fixed scaffolds were washed repeatedly with PBS to remove glutaraldehyde and then air dried overnight. Dry cellular constructs were sputter coated with gold and observed by SEM at an accelerating voltage of 20 kV.

Real Time PCR (RT-PCR)

The effects of the fiber diameter on the expression of ECM proteins namely collagen type I, type III and elastin were evaluated using RT-PCR. At predetermined time points, 7, 14 and 28 days after culture, the cellular constructs were washed with PBS solution and the total RNA from the cells was isolated using Trizol (Gibco BRL, USA) following the procedure described by the manufacturer (Qiagen, 74106, USA). Briefly, 1ml of trizol in total was added to the matrices and maintained at room temperature for half an hour. The trizol digestion solution was collected and RNA extracted by the addition of 0.2ml of chloroform [Fisher, C298-500, USA] and centrifugation at 12,000g for 15 minutes at 4°C. The RNA extracted was stabilized using 70% ethanol (prepared by diluting absolute ethanol [Aaper, USA] with nuclease free water [Ambion, USA]). The RNA was centrifuged using a QIA Shredder Spin Column [Qiagen, USA], and dissolved in RNase free water [Qiagen, USA]. The concentration of the RNA was measured using a spectrophotometer at 260nm. Reverse transcription was performed with MultiScribe reverse transcriptase (Applied Biosystems, Forster City, CA) and random hexamers as per the manufacturer's instruction. The resulting cDNA were then each subjected to quantitative RT-PCR. Gene expression was determined using SYBR-Green for RT-PCR [Applied Biosystems, ABI Prism, 7900 HT Sequence Detector System, 134 USA]. The primers were designed on the basis of published gene sequences (NCBI and Pubmed) and shown in Table 1. Quantitative values were determined by the Delta-Delta method and normalized with the house keeping gene, β -Actin (Statistical significance at $p < 0.05$, $n = 3$) [24].

Statistical analysis

All results were first evaluated using one-way analysis of variation (ANOVA) followed by Tukey's HSD (Honestly Significant Differences) analysis of the differences between groups with a confidence range of 95.00 %.

Results

Fabrication of nonwoven PLAGA Fiber Matrices

PLAGA 50:50 solution in THF:DMF (3:1) were prepared at concentrations of 0.2, 0.225, 0.24, and 0.27 g/mL and electrospun into fiber matrices having fiber diameters in the range of 150–225, 200–300, 250–467 and 500–900 nm respectively. Concentrations of 0.3, 0.35, and 0.42

g/mL resulted in microfiber matrices having fiber diameters in the range of 600–1200, 2500–3000 and 3250–6000 nm respectively. Fiber diameters of electrospun fiber matrices had a Gaussian type distribution. Reported fiber diameters of 150–225, 200–300, 250–467, 500–900, 600–1200, 2500–3000, and 3250–6000 correspond to lower and higher fiber diameter population on the curve. The higher and lower values reported for every fiber diameter range is equivalent to the full width of the Gaussian distribution curve at half the maximum. However, the average values of the fiber diameters were considered to determine statistical significance. Fiber matrices were named as Matrix-1, Matrix-2, Matrix-3, Matrix-4, Matrix-5, Matrix-6, and Matrix-7 based upon increasing fiber diameter for identification purposes throughout the study. Matrix-2 had fiber diameters of 200–300 nm, which overlaps with fiber diameters of Matrix-3 (250–467). For identification purposes average fiber diameter of Matrix-3 350 ± 75 nm was considered. Electrospun nonwoven fiber matrices of thickness between 0.38–0.42 mm were used in this study. SEM micrographs of fiber matrices of varying fiber diameter are presented in Fig. 1. On increasing the polymer concentration, the viscoelastic force supersedes surface tension, and thus bead defects disappear. Scaffolds are distinguished by increasing fiber diameters due to higher cohesive force and reduced stretching of the fibers. This increase is observed to follow a “Power Law” relationship as reported earlier though the value of the exponent differed for different polymer solution systems [25,26]. Increased fiber diameter due to increased polymer concentration from 0.2 g/mL – 0.42 g/mL is satisfied by exponent values in the range of 4.5679 – 4.6134. The fiber diameters of matrix 1 and 2 are similar, and both are significantly different from samples 3 – 7. In addition, fiber diameters of matrices 3–7 are significantly different from one another (Fig. 2). Fiber diameter affects mechanical properties, porosity and surface wettability (hydrophilicity/hydrophobicity). Small fiber diameter accounts for higher surface area, higher tensile properties, lower porosity and lower wettability [27]. These fiber matrices were found to have a tensile modulus from 39.23 ± 8.15 to 79.21 ± 13.71 MPa and porosity ranging from 38–60 % with average pore diameters of 10–14 μ m. The two week, 37°C phosphate buffered degradation profile for the smaller fiber diameter and microfiber matrices showed identical molecular weight loss patterns [27]. The scaffolds retained about 35% of the original molecular weight at day 14.

Human Skin Fibroblast Proliferation, Morphology and Gene Expression

Cell numbers increased on all samples over the time period studied with fiber diameter dependent proliferation behavior noted at all time points studied. Fiber Matrix-3, 4 and 5 showed significantly higher proliferation rate than Matrix-1, 2, 6 and 7 at all the time points. At day 1, 3, 7, 14, and 28 (Fig. 3 B–F) Matrix-3, 4 and 5 showed similar number of cells and measured a significantly higher cell number (> 2.5 , > 3.5 , > 4.5 , > 6 and > 12.5 fold respectively) than the initial seeding. Human skin fibroblasts showed significantly higher proliferation on nonwoven electrospun PLAGA fiber matrices having fiber diameters in the range of 350–1100 nm. Below and beyond this diameter range, significantly reduced proliferation was measured (Fig. 4). Matrix-5 showed highest proliferation rate, and was chosen to present cell survival and morphological studies. Fluorescent microscopy imaging shows live cells (stained green) to have adhered and spread well on the fiber matrices of lower diameter (Fig. 5).

On matrix-1, 2 and 6 (Fig. 6 A, B and F), hSF was observed (using SEM) with a rounded morphology at 1h after seeding; where as the cells acquired a spread morphology on Matrix-3, 4 and 5 (Fig. 6 C, D and E). Cells did not adhere to Matrix-7 (Fig. 6 F) at 1h post-seeding. By day 14, hSF were well spread and formed multiple cell layers on Matrix-3, 4 and 5 (Fig. 7 C, D and E) whereas Matrix-1, 2, 6 and 7 had uncovered surfaces (Fig. 7 A, B, F and G). SEM micrographic observations support the hSF proliferation trend quantified by MTS assay at the time points studied. The morphology and proliferation of hSF on Matrix-4 at various culture time points is presented in Fig. 8. Matrix-4 was chosen as a representative because it showed a higher proliferation rate in that diameter range. At time points 1h, 4h and 12h, cells adhered

and showed well spread morphology (Fig. 8 A, B and C). Cell density increased with increasing culture period from day 1 to day 28 (Fig. 8 C–H). Surface of Matrix-5 was completely covered by hSF on day 14 (Fig. 8 G) and was multilayered by day 28 (Fig. 8 H).

At day 7, collagen type I gene expression by hSF cultured on lower diameter fiber matrices showed significantly higher expression than microfiber Matrix-7 (Fig. 9 A). Collagen I expression decreased on day 14 and it increased up to day 28. Collagen III expression by hSF showed fiber diameter dependency and increased with culture time (Fig. 9 B). Lower diameter fiber matrices, namely Matrix-3–5, resulted in significantly higher collagen III expression than the rest of the fiber matrices at days 14 and 28. Elastin expression remained near constant for all the time points studied (Fig. 9 C). However at day 14, expression of elastin was higher on lower fiber diameter matrices namely Matrix-3–5, which are composed of diameters in the range of 350–1100 nm. Elastin expression did not show any fiber diameter dependency unlike collagen III.

Discussion

The Food and Drug Administration (FDA) has approved PLAGA for a variety of biomedical applications. Its physical properties such as degradation and mechanical profile can be tuned by altering its composition. Varying ratios of copolymers, PLA:PGA namely 85:15, 65:35 and 50:50 were fabricated into thin films and their mechanical properties and degradation profile (data not shown) characterized. PLAGA 50:50 copolymer was found to be suitable for our intended application based on the measured tensile properties and degradation pattern. All the fiber matrices showed suitable tensile properties (tensile modulus from 39.23 ± 8.15 to 79.21 ± 13.71 MPa) for skin grafting applications within the range of normal human skin (15–150 MPa) [28]. Fiber diameter is known to influence wettability of the matrix. It is observed that with the decrease in fiber diameter of the electrospun fibers wettability also decreases. It is speculated that the mats containing smaller fiber diameter exhibit higher degrees of roughness since it is known that surface roughness combined with the intrinsic non-wetting characteristics of a substrate lead to pronounced decrease in wettability [29]. Studies have shown that the cells were adhered, spread, and grown more onto positions with moderate hydrophilicity of the chemogradient PLAGA surface [30]. We also made efforts to measure water contact angles on nonwoven PLAGA scaffolds of varying fiber diameters. Observed values were not consistent and did not show any systematic trend as fiber matrices had uneven surface. However, the observed values were around $55^\circ \pm 4^\circ$ for Matrix-3, 4 and 5 which are in line with the reported values where cells showed improved performance [30]. While control PLAGA film had a water contact angle of $72^\circ \pm 3^\circ$ was relatively hydrophobic than the fiber matrices. The degradation profile is also found to be ideal for skin grafting applications [27]. Following 2 week degradation SEM micrographs revealed the intact macro structures for both the fiber matrices (data not shown) and these results are similar to the earlier report [27]. These porous electrospun fiber matrices possess suitable physico-chemical properties for tissue engineering applications [6]. PLAGA scaffolds composed of varying fiber diameters fabricated using an electrospinning set up were evaluated *in vitro* using hSF.

The observed proliferation trend on PLAGA fiber matrices showed fiber dependency (Fig. 3 and Fig. 4). Human skin fibroblast proliferation on lower diameter fibers in the diameter range of 150–225 and 200–300 nm, and microfibers in the diameter range of 2500–3000 and 3250–6000 nm was significantly lower than fiber matrices having fiber diameters in the range of 250–467, 500–900 and 600–1200 nm. Several cell studies using electrospun lower diameter fiber matrices have shown better cellular response in a particular diameter range [21,22,31].

Initial hSF adhesion after 1h of seeding as visualized by SEM in Fig. 6 supports the observed fiber diameter dependent proliferation trend at early stages. Cells acquired rounded

morphology on lower fiber diameter matrices of 150–225 and 200–300 nm, and microfibers in the diameter range of 2500–3000 nm. Rounded morphology is an indication of reduced interaction between cells and topographic features. Cells adopted well spread morphology on lower diameter fiber matrices having diameters 250–467, 500–900 and 600–1200 nm, which indicated a favorable interaction between cells and nanostructures [32,33]. These observations were further supported by cell survival and morphology studies presented as fluorescence images (Fig. 5). Representative lower fiber diameter matrix of fiber diameter 600–1200 nm showed more number of viable cells with well spread morphology on day 28 than day 14. Different stages of hSF proliferation on a lower fiber diameter matrix with fiber diameter in the range of 500–900 nm were visualized by electron microscopy (Fig. 8). Cells adhered well, showed progressive growth, well spread morphology, and by day 28 scaffold surfaces were covered with a multilayer of cells. SEM micrographic observations support the hSF proliferation trend measured by the MTS assay at all the time points.

In the present study we have quantified collagen type I, type III and elastin gene expression by hSF cultured on nonwoven fiber matrices of varying fiber diameter at different culture times using Real Time-PCR. Native tissue ECM consists of a fibrous network of collagen and elastin as the major structural proteins. These structural proteins impart mechanical properties to the tissue. During the process of wound healing, growth factors stimulate fibroblasts to synthesize new collagen, elastin and glycoproteins to replace the provisional wound and form scar tissue. The strength of the repair tissue at the wound site is ultimately based on the composition of the ECM; more specifically the type and organization of collagen and elastin [34–36]. Wound healing process is characterized predominantly by fibroblast proliferation and ECM synthesis and remodeling into a highly organized architecture [37]. Previous studies have shown that collagen gene expression is regulated in a time-dependent fashion [38]. In the early phase of wound healing the expression of collagen Type I and Type III is coordinately increased throughout the entire dermis [39]. Fibroblasts recruited to the wound site establish continuity and synthesize collagen, proteoglycans and other ECM components. Collagen Type III expression is found to be predominant in granulation tissue which is mechanically poorer than the surrounding native dermis [37]. In our present study we observed a similar collagen gene expression trend with time; collagen Type III expression is many fold greater than collagen type I gene expression during the initial time points assayed. During the later time point, collagen type I gene expression is increasing significantly. Elastin fiber network provides skin the ability to extend and recoil under tension. Studies have demonstrated reduced tensile strength in scar tissue following cutaneous wounding, due to decreased amounts or breakage of elastic fibers [35,36]. Elastin gene expression remained largely constant on most matrices throughout the length of the culture period studied. More pronounced changes in elastin gene expression may occur at later time points synonymous with the remodeling stage of wound repair. The dermal components of artificial skin substitutes lack an organized elastic fiber network, which may contribute to excessive contraction and scarring after post-grafting [36]. Expression of collagen and elastin genes by dermal fibroblasts on fiber matrices of varying fiber diameter is in line with the earlier *in vivo* observations.

Conclusions

Fiber matrices with appropriate mechanical strength, degradation pattern and porosity were fabricated via electrospinning to develop skin grafts. In this study we optimized the fiber diameters suitable for developing artificial skin grafts by seeding human skin fibroblasts. Fibroblast proliferation showed fiber diameter dependency and lower fiber diameter matrices in the diameter range of 350–1100 nm showed significantly higher proliferation rate than the fiber matrices below and beyond this fiber diameter range. Human skin fibroblast showed a well spread morphology and formed cell multi-layers on the scaffolds after 28 days in culture.

Studies are underway to investigate the efficacy of these fiber matrices to regenerate skin in suitable animal models.

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Figure 1.

SEM of electrospun nonwoven PLAGA fiber matrices at a magnification of 5000X using constant electrospinning parameters of 20G needle, voltage gradient 1kV/cm and flow rate of 2 mL/h. The concentration and average fiber diameters of different samples are (A) Matrix-2: 0.225 g/mL, 200–300 nm, (B) Matrix-3: 0.24 g/mL, 250–467 nm, (C) Matrix-4: 0.27 g/mL 500–900 nm, (D) Matrix-5: 0.3 g/mL, 600–1200 nm, (E) Matrix-6: 0.35 g/mL, 2500–3000 nm, and (F) Matrix-7: 0.42 g/mL, 3250–6000 nm.

Figure 2.

Comparison of PLAGA fiber diameters resulted from electrospinning of various polymer concentrations from 0.2 g/mL to 0.42 g/mL keeping all the process parameters constant at 20G, 1kV/cm and 2 mL/h. Nonwoven fiber scaffolds are named as Matrix-1 to Matrix-7 based on the increasing fiber diameter (* indicates the statistical significance as compared to previous group).

Figure 3.

Proliferation of human skin fibroblasts (hSF) seeded on nonwoven electrospun PLAGA fiber matrices of varying fiber diameters. At each time point quadruplicate samples were measured and comparisons were made within the group (* and # indicates the statistical significance within the group). Where (A) hSF proliferation on Sample-1 to Sample-7 and tissue culture poly(styrene) (TCPS) as control at all studied time points. (B) hSF proliferation on day-1, (C) hSF proliferation on day-3, (D) hSF proliferation on day-7, (E) hSF proliferation on day-14, and (F) hSF proliferation on day-28. The hSF proliferation increased significantly on Matrix-3, 4 and 5 when compared to Matrix-1, 2, 6 and 7.

Figure 4.

Effect of fiber diameter on hSF proliferation. At 14 day time point cells showed better proliferation on the nonwoven fiber matrices diameters ranging between 350–1100 nm when compared with other fiber diameters below and above the said range. At all the time points studied hSF showed similar proliferation trend.

Figure 5.

Cell survival and morphology of hSF seeded on nonwoven electrospun PLAGA fiber matrices was determined by viability/cytotoxicity assay. Live cells appear as fluorescent green colour and dead cells as fluorescent red. Cell survival was determined on (A) Matrix-5 14 day (B) Matrix-5 28 day (C) Matrix-5 14 day at higher magnification. Cells maintained a normal morphology and survived on all the scaffolds. Similar results were also observed on the time points of 7 and 28 days (data not shown).

Figure 6.

Analysis of attachment and morphology of hSF seeded on nonwoven electrospun PLAGA fiber matrices at an initial time point of 1 h. SEM micrographs of (A) Matrix-1, (B) Matrix-2, (C) Matrix-3, (D) Matrix-4, (E) Matrix-5, (F) Matrix-6, and (G) Matrix-7 were taken at a magnification of 400X. Rounded morphology of hSF was observed on Matrix-1, 2 and 6. Spreading of hSF was observed on Matrix- 3, 4 5 and no cells were detected on Matrix-7.

Figure 7.

SEM micrographs of hSF seeded fiber matrices at a time point of 14 days. Micrographs of (A) Matrix-1, (B) Matrix-2, (C) Matrix-3, (D) Matrix-4, (E) Matrix-5, (F) Matrix-6, and (G) Matrix-7 were taken at a magnification of 400X. hSF proliferation was observed on fiber matrix surfaces; Matrix-3,4 and 5 showed multilayer of cells, wherein Matrix-6 and 7 have still lot of uncovered surfaces.

Figure 8. Attachment and proliferation of seeded hSF on Matrix-4 at various time points of (A) 1h, (B) 4 h, (C) 12 h, (D) 3 day, (E) 7 day, (F) 14 day, and (H) 28 day. Progressive growth of hSF was observed with increasing time at day 14 and achieved complete nanofiber surface coverage, and at 28 days the cells formed multilayers.

Figure 9.

Gene expression by human skin fibroblasts seeded on nonwoven electrospun PLAGA fiber matrices of varying fiber diameters for different periods of time, determined using a real time PCR. Quantitative values of (A) Collagen I, (B) Collagen III and (C) Elastin were determined by the Delta-Delta method and normalized with the house keeping gene, β -Actin. At each time point triplicate samples were measured and comparisons were made within the group (* indicates the statistical significance at $p < 0.05$). Collagen III showed a fiber diameter dependency in its expression and Matrix-3–5, diameters in the range of 350–1100 nm resulted in increased collagen III expression on day 14 and 28.

Table 1

The sequences for forward (sense) and reverse (anti sense) gene specific primers for hSF used in RT-PCR amplification.

Genes	Sequence Name	Primer	Sequences	Base No
β-Actin	HsActinB4	HsActinB4-997F	CCCTGGCACCCAGCAC	16
		HsActinB4-1067R	GCCGATCCACACGGAGTAC	19
Collagen Type I	NM 000088	COL1A1-48F	TGGTGCAGCTGGTCTTCCA	19
		COL1A1-139R	CACGGACGCCATCTTTGC	18
Collagen Type III	NM 000090	COL3A1-118F	GATGTGCAGCTGGCATTCC	19
		COL3A1-218R	CCACTGGCCTGATCCATGTAT	21
Elastin	NM 000501	elastin-309F	CCAAAGCCGCCAGTTT	17
		elastin-454R	AAGGCCAGCAGCACCGTAT	19