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Research article

Electrospinning of nanofibrous polycaprolactone (PCL) and collagen-blended polycaprolactone for wound dressing and tissue engineering

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Abstract

Fabrication of nanofibrous biomaterials based on natural materials through various techniques is a popular research topic, particularly for biomedical applications. Electrospinning, a well-established technique for nanofiber production has also been extended for producing nanofibrous structures of natural materials that mimic natural extracellular matrix of mammalian tissues. Collagen nanofiber production utilizes hexafluoro propanol (HFP) as a solvent for electrospinning. A novel cost-effective electrospun nanofibrous membrane is established for wound dressing and allogeneic cultured epidermal substitute through the cultivation of human dermal keratinocytes for skin defects. Several synthetic polymers such as polycaprolactone (PCL) are generally electrospun for tissue engineering applications because of their remarkable mechanical stability and slow degradation rates. The large surface area of the polymer nanofibers with specific modifications facilitates cell adhesion and control of their cellular functions. The objectives of this study were to optimize fabrication parameters of electrospun nanofibrous membranes from biodegradable PCL and collagen-blended nanofibrous membranes to combine mechanical integrity and spinnability of PCL with high biocompatibility of collagen, and to examine keratinocyte attachment, morphology, proliferation, and cell-matrix interactions. Results prove that the porous nanofibrous PCL and modified PCL-blended collagen nanofibrous membranes are suitable for the attachment and proliferation of keratinocytes, and might have the potential to be applied as wound dressing as well as in tissue engineering as an epidermal substitute for the treatment of skin defects and burn wounds.

Keywords: *Scaffold, electrospinning, collagen, polycaprolactone, tissue engineering.*

1. Introduction

Skin is the largest organ of human body and serves as a barrier between the human body and the environment. It protects the body against pathogens and microorganisms. Accordingly, it is under the threat of microbial, thermal, mechanical and chemical threats. In the past 30 years, many studies were conducted to develop substitutes which mimic human skin [1,2]. These skin substitutes were developed for use in clinical applications

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and to support the healing of acute and chronic wounds, and also as complex test systems for dermatological and or pharmacological applications [3–6]. In this process, tissue engineering is used to construct these skin models. In brief, various biological and synthetic materials are used to produce cell carriers (scaffolds) and these are seeded and cultivated with cells required by the target tissue. To this two-component system, bioactive agents that accelerate growth and increase differentiation of cells are added to hasten tissue formation. An ideal scaffold should have appropriate physical and chemical structure and surface properties to allow cell attachment, proliferation and differentiation as well as physiologically correct cellular behaviors [7,8].

Burns, traumas and circulatory system diseases may result in massive skin tissue loss. Occasionally these tissue losses can affect a large portion of the patient's body, and therefore, be larger than the size of the material that can be harvested from the patient's own body (autograft) [9,10]. The current models developed by using advanced materials science and engineering methods are capable of mimicking the entire epidermis; but models mimicking also the complete organization of the dermis layer have not been developed yet. These cases are considered as important clinical issues that have not been fully solved [11,12].

The goal of wound dressing is to produce an ideal structure that will give higher porosity for cell motility, nutritional and oxygen needs and also serve as a good barrier against pathogens. To reach this goal, the wound dressing materials must be selected carefully and the structure must be controlled to confirm that it has good barrier properties and oxygen permeability [13,14]. Electrospun nanofibrous membranes have been shown to support good and immediate adherence to wet wound surfaces and attain uniform adherence to the wound surface without any fluid accumulation. Rate of epithelialization had increased and the well-organized dermis in electrospun nanofibrous membranes provided a good support for wound healing [15,16].

By nature, human organs are constituted by fibrous extracellular matrix (ECM) made up of nanofibers; and replacing or mimicking this microstructure is a challenging task. Electrospinning is one of the most established processes for producing nanofibrous structures that mimic the ECM. It has also been reported that nanofibrous materials obtained by electrospinning improve tissue regeneration and decrease scar formation [17,18].

In electrospinning, nanofibers are obtained as a result of the electrostatic charges overcoming the surface tension of polymer droplet and subsequent deposition of these nanofibers onto a collector to obtain nonwoven matrices [19,20]. The parameters influencing the process are broadly classified into three groups, namely solution properties (viscosity, conductivity, surface tension, polymer molecular weight, dipole moment, and dielectric constant); controlled variables (material flow rate, electric field strength, distance between tip and collector, needle tip design, collector composition, and geometry); and ambient conditions (temperature, humidity, and air velocity) [21–24]. Even though lots of synthetic materials are being electrospun, the major bottleneck in usage of synthetic materials for biomaterial production lies in their limited biocompatibility. Materials of natural origin are likely to overcome this problem and can be used for the production of highly biocompatible biomaterials [25–27]. Collagen, a well-studied natural structural protein has always been a choice as a substrate for tissue engineering, because of its biocompatible and biodegradable properties [28–31]. Polycaprolactone (PCL) is a long used soft- and hard-tissue compatible material for applications including resorbable sutures, drug delivery systems and is recently

developed as a bone graft substitute. Its prospective use as a supportive scaffold material has been reported for a variety of tissue engineering applications [32–35]. Combining the advantages of the two materials and using electrospinning for production of ECM biomimicking microstructure, collagen-PCL composite nanofibers have been shown to be promising candidates for tissue engineering applications [36–38]. This study has shown that, in addition to fibroblasts, osteoblasts and other cell types reported in the literature, these composites also support human keratinocyte attachment and proliferation, which substantiates them as good candidates for wound dressing and epidermal tissue engineering applications.

2. Materials and Methods

2.1. Materials

Dulbeco's modified Eagle's medium (DMEM Ham's F12), fetal bovine serum (FBS), antibiotics, trypsin–ethylenediaminetetraacetic acid (EDTA) were purchased fromHyclone, (ABD), cell culture plates were purchased fromCellstar, (Germany); hexafluoro propanol (HFP), polycaprolactone (PCL) (4407444, molecular weight (MW) 80 000) and collagen from rat tail (C7661), sodium cacodylate, glutaraldehyde, osmium tetroxide and hexamethyldisilazane (HMDS) were purchased from Sigma Aldrich (U.S.A.), and dimethylformamide (DMF) and dichloromethane (DCM) were purchased from Merck (Germany).

2.2. Apparatus

An electrospinning setup (Inovenso, Turkey) consisting of a programmable infusion pump, high voltage apparatus, a 5-mL syringe with a 21-G needle and a stationary aluminum plate as collector was used for the production of nanofibers (Fig. 1).

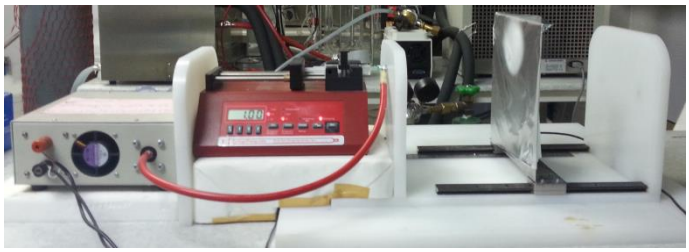
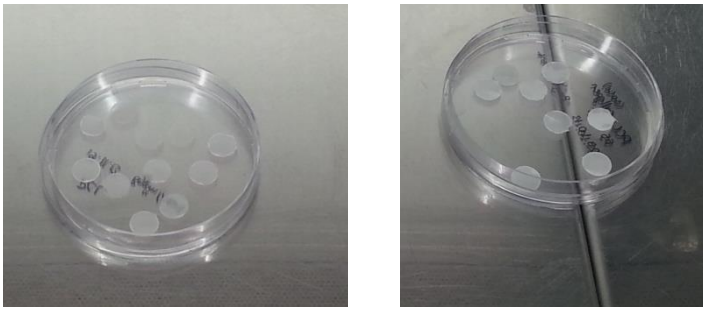


Fig. 1 Electrospinning device

2.3. PCL Scaffold Production

PCL solutions with concentration of 12 wt% were prepared by dissolving PCL in dimethylformamide (DMF) and dichloromethane (DCM) (1:1). The mixtures were stirred overnight. 5 mL syringes were filled with prepared solutions and syringes were placed in the infusion pump device. The production parameters were optimized to be 1 mL/h flow rate, 15.7 kV voltage difference and 15 cm distance between the needle tip and the collector plate. Produced fibers were collected on glass lamellae which were fixed on the plate in order to be used in cell culture experiments (Fig. 2).



(a) (b)

Fig. 2 Scaffolds on cover slips: (a) PCL, and (b) PCL/collagen scaffolds on cover slips

2.4. PCL/Collagen Scaffold Production

PCL and type-I collagen from rat tail with concentration of 55:25 were prepared by dissolving PCL and collagen in hexafluoro-propanol (HFP). The mixture was stirred overnight. 5 mL syringes were filled with prepared solutions and syringes were placed in the infusion pump device. The production parameters for the composite were optimized to be 2 mL/h flow rate, 15 kV voltage difference and 12 cm distance between the needle tip and the collector plate. Similar to the PCL fibers, produced composite fibers were also collected on glass lamellae fixed on the plate (Fig. 2).

2.5. Fourier Transform Infrared (FTIR) Spectroscopy Analysis

The FTIR spectroscopy measurements in a 600-4000 cm 1 range were performed on a FT-IR spectrometer (Frontier, Perkin-Elmer) using the multiple internal reflectance (MIR) technique with a KRS-5 crystal (458) and a TGS detector. The spectra were obtained at a 2 cm-1 resolution, 100 scans.

2.6. Scaffold Sterilization

Before starting the in vitro experiments both sides of the PCL and PCL/collagen nanofibers on coverslips were sterilized with UV light for 45 min.

2.7. Cell Culture

HS2 (human dermal keratinocyte) cell line obtained from Animal Cell Culture Collection 183 (HUKUK, Sap Institute, Ankara, Turkey) were used. Keratinocytes were seeded on standard polystyrene tissue culture flasks and cultured in an optimized medium for preservation of keratinocyte phenotype, which is composed of DMEM/Ham's F12 1:1, 10% fetal bovine serum and1% penicillin/streptomycin (10,000 units/mL penicillin-10,000 μg/mL streptomycin). The medium was changed every other day. At confluence, cells were re-suspended by trypsin-EDTA and passaged. In all experiments keratinocytes between passages 4 and 8 were used.

2.8. HS2 Cell Culture on PCL and PCL/Collagen Nanofibrous Matrices

Cells were detached from the flask surface by treatment with 0.25% trypsin for 5 min at 37°C. After detachment, trypsin was deactivated with serum and cells were collected by centrifugation. After determination of the cell number, keratinocytes suspended in 50 μL

medium were seeded at a density of 5×103 cells/scaffold onto each nanofiber scaffold placed in 24-well plates for the determination of attachment, morphology and growth profile. After seeding, the scaffolds were incubated for cell attachment in a CO_2 incubator for 1 hr, then at the end of 1hr the volume of the medium was completed to 500 µL under sterile conditions, and the scaffolds were cultured at 37°C in a 5% CO_2 humidified environment for up to 48 hrs.

2.9. Cell Proliferation

To evaluate cell metabolism on the nanofibrous scaffolds, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich,Germany) assay was performed in triplicate after 24 and 48 hrs on nanofibers where the initial cell number was 5×103 cells/scaffold. The MTT assay is dependent on the cellular reduction of MTT [3-(4, 5-dimethylthialzol-2-yl)-2, 5-diphenyltetrazolium bromide], by the mitochondrial dehydrogenase enzymes of viable cells, to a blue formazan product which can be measured by a spectrophotometer. The amount of purple formazan crystals formed is proportional to the number of viable cells. MTT solution in DMEM medium (1:9) was added onto each well of a 24-well plate and they were incubated at 37°C and 5% CO_2 for 3 hrs. After 3 hrs, the MTT solution was aspirated from the well and replaced with dimethyl sulfoxide (DMSO), which solubilized the formazan crystals. The solutions were transferred to a 96-well plate and the absorbance of MTT–formazan product released was measured at 590 nm (690 nm reference) on a microplate reader (Moleculer Devices-Versa Max). Cells seeded on standard 24-well polystyrene dishes were used as control, and the results were reported as percent ratio of their absorbance to the controls. The statistical analysis was performed using one-way analysis of variance (ANOVA) method. Post-hoc multiple comparisons of the mean of independent groups were made using Tukey test at statistically significance value, $p < 0.05$.

2.10. SEM Analysis of the Scaffolds

The morphology of the electrospun scaffolds was examined by scanning electron microscopy (QUANTA FEG 250). Dry scaffolds were collected from six distinct regions within a mesh, sputter coated with gold–palladium to reduce charging and produce a conductive surface, and imaged in secondary electron mode at 5 kV. Images were collected, and the void space calculated from five random images of each scaffold type, and the diameter of at least 50 fibers from each scaffold type was assessed quantitatively via NIH ImageJ software (freeware http://rsb.info.nih.gov/ij/).

2.11. Water Contact Angle Measurements

Biomaterials are in contact with water, blood, and other body fluids during their lifetime. Thus, while intending to produce materials for biomedical applications such as scaffolds for wound healing or skin regeneration, it is essential to characterize them for their wettability. Surface contact angle measurements for the electrospun PCL and PCL/collagen were done by assessing the contact angle made by water on the surface of the electrospun matrices by a video contact angle system (KSV Instruments).

2.12. SEM Analysis for Cell Seeded Scaffolds

The media were aspirated from culture wells; the scaffolds were washed with 0.1M sodium cacodylate buffer and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate at 4°C for 2 hrs, and post fixed in 1% osmium tetroxide in 0.1M cacodylate for 90 min in the

dark. Following a buffer rinse, the samples underwent gradual dehydration in an ethanol series (50%, 70% ethanol for 5 min followed by 80%, 95%, 100%, and 100% for 10 min). Finally, samples were placed in HMDS for 45 min, allowed to dry overnight, and stored in a desiccator. Samples were sputter-coated with gold-palladium for 2 min prior to imaging by scanning electron microscopy (QUANTA FEG 250).

2.13. Cytoskeletal Observation by Immunofluorescence Staining

Keratinocyte seeded nanofiber scaffolds were stained with the nuclear stain DAPI (Invitrogen, D1306, ABD) and filamentous actin stain Alexa Fluor 488 conjugated Phalloidin, an actin-binding toxin isolated from Amanita phalloidses (Life Technologies). After fixation in 4% formaldehyde solution for 15 min, specimens were rinsed with phosphate buffered saline (PBS) and then DAPI solution (diluted 1:1000 in PBS), and Alexa Fluor 488 Phalloidin solution (diluted 1:200 in PBS) were applied onto the scaffolds which were incubated at 37°C in the dark for 45 min. Afterwards, specimens were rinsed with PBS and examined using a fluorescence microscope (Leica DMIL, Germany) with near-ultraviolet WU filter (330–385 nm).

3. Results

3.1. Scaffold Characterization

Before starting the in vitro experiments, the physical characteristics of the scaffolds that affect cellular behavior, such as surface chemistry, porosity and fiber diameter distribution of the nanofibers and surface wetting properties were determined.

3.1.1. SEM analysis

Fiber morphology of the electrospun samples was determined via scanning electron microscopy operating at 5 kV. The process parameters were optimized so that no beading was observed on either type of scaffolds (Fig. 3). The non-woven fibers were collected in a random alignment, and a highly porous structure with interconnected porosity was achieved. Taking an average of five measurements for each image, the void space represented 84±4% for PCL and 81±3% for PCL/collagen scaffolds. Average fiber diameter was estimated to be 367±115 nm with diameters ranging 190–640 nm for PCL scaffolds, and to be 328±92 nm with diameters ranging 188–660 nm for PCL/collagen scaffolds (Fig. 4). PCL scaffolds showed virtually more homogeneous distribution of fiber diameters, but there was no statistically significant difference between the average fiber diameters of the two types of scaffolds.

3.1.2. Fourier transform infrared (FTIR) spectroscopy analysis

Surface chemistry of the two types of scaffolds was characterized by Fourier Transform Infrared (FTIR) spectroscopy (Frontier, Perkin-Elmer) As seen in Fig. 5, characteristic infrared bands of PCL can be observed as: asymmetric CH_2 stretching at 2943 cm^{-1}, symmetric CH_2 stretching at 2866 cm^{-1}, carbonyl (C=O) stretching at 1721 cm^{-1}, C–O and C–C stretching in the crystalline phase at 1721 cm^{-1}, asymmetric COC stretching at 1239 cm^{-1}, and O–C–O stretching at 1190 cm^{-1} [39]. The proof of the existence of collagen in PCL/collagen spectrum are the two peaks due to stretching of amide-I and amide-II at 1646 cm^{-1} and 1537 cm^{-1}, respectively [40].

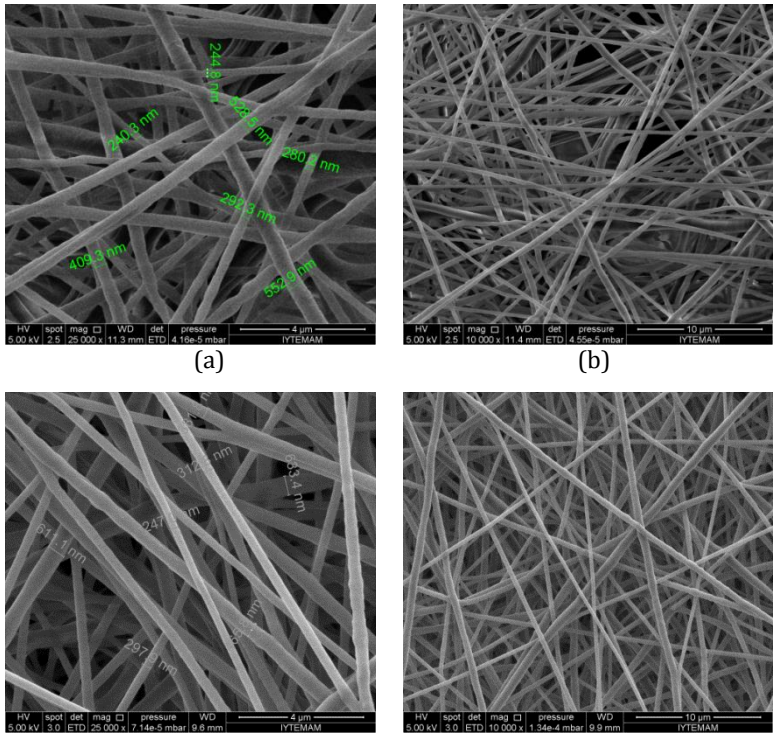


Fig. 3 SEM micrographs of PCL and PCL/collagen nanofibers: (a)PCL scaffold, (b)PCL scaffold overall structure, (c)PCL/collagen scaffold and (d)PCL/collagen scaffold overall structure

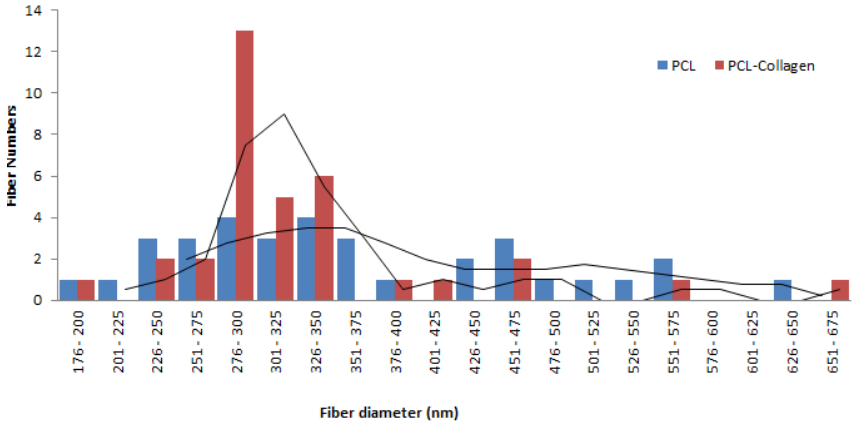


Fig. 4 Fiber diameter distribution

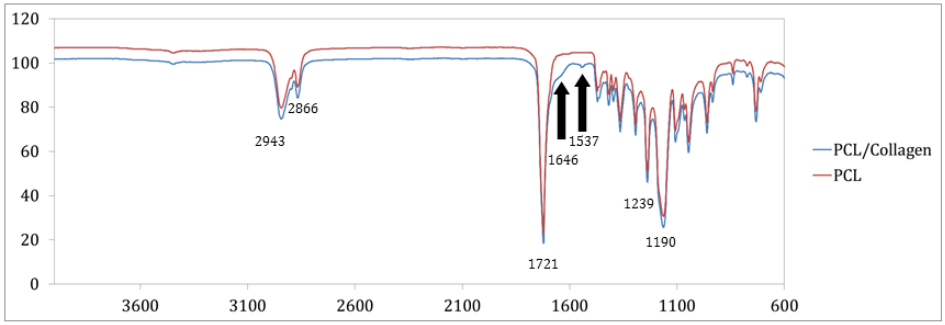


Fig. 5 FTIR analysis of PCL/collagen scaffolds

3.1.3. Surface contact angle

The average water contact angle was measured as 136.32°±4 for the PCL nanofibrous scaffolds and 111.81°±4 for the PCL/collagen nanofibrous scaffolds. PCL is known to have a hydrophobic nature, and upon blending with collagen, a well-known hydrophilic protein with high water treatment capacity, the hydrophilicity increased significantly as observed by the decrease in contact angle [52].

3.2. HS2 Cell Culture on PCL and PCL/Collagen Scaffolds

3.2.1. Cell proliferation analysis (24 hrs, 48 hrs)

There was no significant difference between the scaffold types and the control polystyrene surfaces in terms of initial cell attachment and metabolic activity. The MTT results revealed that all scaffolds favored human keratinocyte cell attachment and proliferation during the initial 48 hrs. Since tissue culture polystyrene flasks are optimized for cell attachment and proliferation, they are usually considered as the positive control for cell attachment. Our MTT values show that PCL and PCL/collagen surfaces favor cell attachment as well as standard surfaces (Fig. 6).

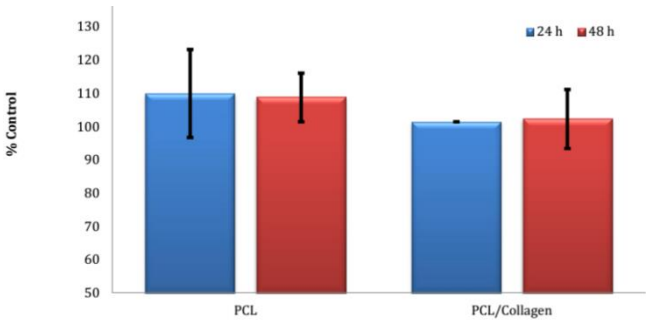
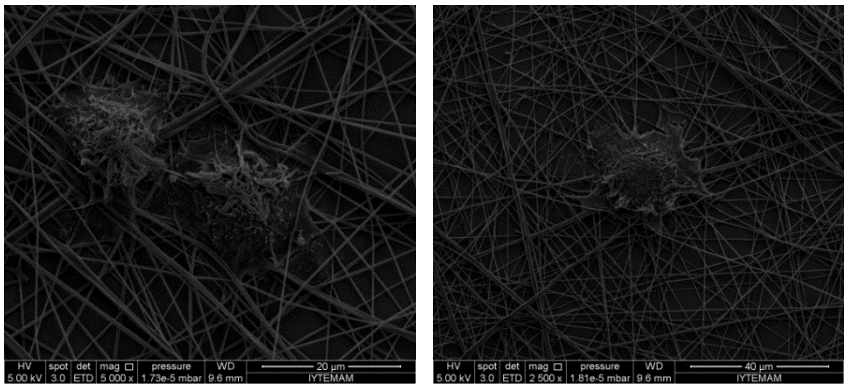


Fig. 6 HS2 human epidermal keratinocytes cell viability on scaffolds

3.2.2. SEM analysis

SEM micrographs of the keratinocytes seeded on the nanofibrous scaffolds predominantly showed a single-cell attachment characteristic with a significant amount of cell spreading on the scaffold surfaces after 48 hrs. Keratinocytes showed typical,

relatively symmetrical epithelial cell morphology with lateral dimensions ranging between 25–55 μm. Cells show distinct filopodia showing strong attachment onto fiber surfaces (Fig. 7).

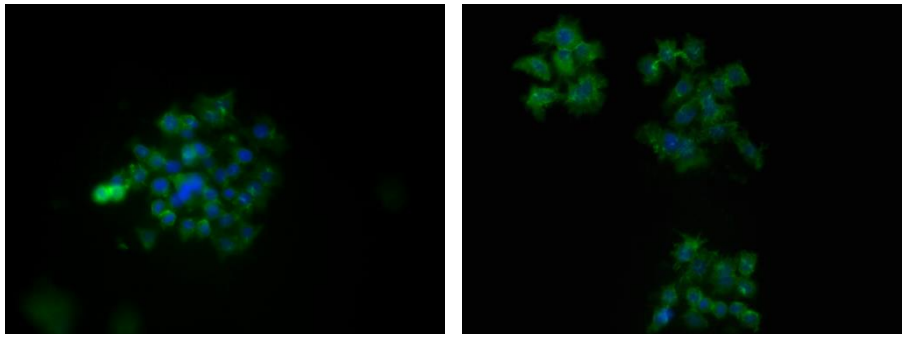


(a) (b)

Fig. 7 SEM micrographs keratinocyte cells on nanofibers after 48 hrs incubation: (a) PCL and (b) PCL/collagen scaffold

3.2.3. Fluorescent microscopy

Actin staining with phalloidin showed diffuse actin polymerization with few stress fibers on the nanofibrous scaffolds. Actin filaments were mostly concentrated at the membrane periphery, and particularly at cell-cell junctions (Fig. 8).



(a) (b)

Fig. 8 Fluorescence micrograph keratinocyte cells on patterned collagen films stained with DAPI and Alexa Fluor 488 Phalloidin: HS2 cells on (a) PCL and (b) PCL/collagen scaffold

4. Discussion

Nanofibrous scaffolds were successfully produced using PCL and PCL/collagen by electrospinning technique. Nanofibrous structures have many advantages that make them well suited for wound healing and tissue engineering applications. Besides mimicking the architecture of natural extracellular matrix, higher surface area to volume ratio of nanofibers leads to strong cellular attachment.

Major drawback of electrospun scaffolds is the lack of cell infiltration due to small pore size, and the difficulty of producing three dimensional structures. In this respect, electrospinning is a good choice for production of scaffolds for dermal/epidermal tissue engineering and wound healing, since the skin has a naturally layered structure with distinct cell types in different layers [1,2]. Electrospun nanofibrous membranes provide high permeability together with a carrier function for the epidermal cells while acting as a barrier for pathogens, mimicking the function of basal membrane beneath the epidermal layer [17,18].

PCL was chosen for its high mechanical strength, spinnability and low cytotoxicity [32–35], while collagen was chosen for its well established biocompatibility [28–31].. Combining the advantages of the two materials and using electrospinning for production of ECM biomimicking microstructure in this study has shown that, in addition to fibroblasts, osteoblasts and other cell types reported in the literature, these composites also support human keratinocyte attachment and proliferation, which substantiates them as good candidates for wound dressing and epidermal tissue engineering applications.

FTIR analysis revealed successful incorporation of collagen protein within the PCL matrix evident from the amide-I and amide-II peaks observed only on PCL/collagen scans. SEM micrographs show that non-woven fibers were collected in a random alignment, and a highly porous structure with interconnected porosity was achieved. By image analysis, the void space was found to be 84±4% for PCL and 81±3% for PCL/collagen scaffolds. This high, interconnected porosity is important to achieve nutritional and oxygen permeability. The preferred porosity of tissue engineering scaffolds used for cellular penetration should generally be within the range of 70–90% [41]. Water contact angle measurements affirmed that addition of collagen in PCL decreased water contact angle substantially, thereby increased hydrophilicity of the surface. The presence of collagen has been shown similarly to significantly improve the water affinity and water uptake capacity of synthetic polymers in the literature [42,32]. Both nanofibrous topography and hydrophilicity are extremely important for initial cell attachment and cell-biomaterial interactions [5,11,17,18]. Although no significant difference was observed between the two types of scaffolds in terms of initial cell attachment, cell proliferation and initial cell morphology, both scaffolds were shown to support cell attachment and growth as well as standard tissue culture optimized polystyrene surfaces. SEM micrographs revealed that keratinocytes showed typical, relatively symmetrical epithelial cell morphology with lateral dimensions ranging between 25–55 μm. Epidermal keratinocytes represent the major cell type of the epidermis, making up about 9 % of the cells. Keratinocytes showed strong attachment onto fiber surfaces extending several filopodia. Filopodia are rodlike extensions of 0.1–0.2 μm diameter having a distinct actin architecture and internal geometrical organization. Filopodia form focal adhesions with the substratum, linking it to the cell surface. Many types of migrating cells display filopodia, particularly in their spherical state prior to spreading, which are thought to be involved in both sensation of chemotropic and topographic cues [43,44]. This suggests an important role of filopodia in mediating initial adhesion events and in exploring environmental features. On flat surfaces, initial filopodia are known to disappear quickly within hours during spreading, and leaving their places to lamellapodia [44,45]. It is clearly evident from our observations and from recent literature [46,47], on surfaces with nanotopography, filopodia are stabilized forming strong attachments; they interact with nanofibrillar structures, enable cell orientation and spreading on nanofibrils, and subsequently bend and align them by traction forces.

The cytoskeleton of eukaryotic cells is composed of three filamentous networks: actin filaments (F-actin), microtubules and intermediate filaments. F-actin is concentrated just beneath plasma membrane and is the major cytoskeletal component of lamellipodia and filopodia [47]. It is tightly linked with the plasma membrane and plays a key role in cell motility and mechanical resistance. Actin also generates traction forces against a substrate and is also involved in cell-cell linkages *via* cadherins [47,49]. Cell-cell junctions are especially important for keratinocytes as well as other epithelial and endothelial cells that line tissue surfaces and therefore required to form cohesive cell sheets to resist mechanical challenges and maintain tissue integrity. Parallel to our SEM observations of cell morphology, phalloidin staining of the keratinocytes on PCL and PCL/collagen nanofibrous scaffolds revealed that actin was localized predominantly at cell-cell junctions, where filopodia are mainly located, with some stress fibers in the cytoplasmic region of the cells. Unstructured, diffuse distribution of actin in the cytoplasm is typical for non-confluent keratinocytes. [47,48]. Concentration of actin on the cell periphery and at cell-cell junctions reveal that PCL and PCL/collagen nanofibrous scaffolds support cellular interactions among keratinocytes and direct them for the formation of strong cell sheets required for wound healing and epidermal tissue engineering [50,51].

Topography sensing is one of the most fundamental mechanisms that cells exploit to interact with their environments, either during development, wound healing or metastatic invasion, and therefore must be explored thoroughly for biomaterial evaluations. Any nanofiber that is free to move and align with filopodia will evoke a different mechanosensation than bulk materials or interconnected fiber networks [46]. This is potentially significant in many biomedical applications, since physically or enzymatically cleaved ECM fibers are present in wound sites and pathological tissues, while interconnected ECM fiber networks dominate in healthy tissues.

5. Conclusions

PCL and PCL/collagen nanofibrous scaffolds were successfully produced by electrospinning. Although no significant differences were observed between pure PCL scaffolds and scaffolds containing collagen at early stages up to 48 hrs, both surfaces supported human keratinocyte attachment and proliferation *in vitro*. Observations on cell morphology and cytoskeletal staining revealed that tested nanofibrous surfaces helped keratinocytes maintain their phenotype by highly modulated cell-surface interactions through stable filopodia and strong cell-cell attachments. Therefore electrospun PCL and PCL/collagen non-woven nanofibrous scaffolds are shown to be strong candidates for wound healing and tissue engineering applications; and longer duration experiments with possible co-culturing of different cell types constructing the epidermis and dermis layers of the skin are proposed.

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