

Research article

POLY(L-LACTIDE-CO-GLYCOLIDE) THIN FILMS CAN ACT AS AUTOLOGOUS CELL CARRIERS FOR SKIN TISSUE ENGINEERING

ALEKSANDRA ZUBER¹, JULIA BOROWCZYK¹, ELIZA ZIMOLAG¹,
 MALGORZATA KROK², ZBIGNIEW MADEJA¹, ELZBIETA PAMULA²
 and JUSTYNA DRUKALA^{1, *}

¹Laboratory of Cell & Tissue Engineering, Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology and Malopolska Centre of Biotechnology, Jagiellonian University, Krakow 30-387, Poland, ²Department of Biomaterials, Faculty of Materials Science and Ceramics, AGH University of Science and Technology, Krakow 30-059, Poland

Abstract: Degradable aliphatic polyesters such as polylactides, polyglycolides and their copolymers are used in several biomedical and pharmaceutical applications. We analyzed the influence of poly(L-lactide-co-glycolide) (PLGA) thin films on the adhesion, proliferation, motility and differentiation of primary human skin keratinocytes and fibroblasts in the context of their potential use as cell carriers for skin tissue engineering. We did not observe visible differences in the morphology, focal contact appearance, or actin cytoskeleton organization of skin cells cultured on PLGA films compared to those cultured under control conditions. Moreover, we did not detect biologically significant differences in proliferative activity, migration parameters, level of differentiation, or expression of vinculin when the cells were cultured on PLGA films and tissue culture polystyrene. Our results indicate that PLGA films do not affect the basic functions of primary human skin keratinocytes and fibroblasts and thus show acceptable biocompatibility *in vitro*, paving the way for their use as biomaterials for skin tissue engineering.

Keywords: Burns, Skin regeneration, Wound healing, Keratinocytes, Fibroblasts, Biomaterials, PLGA, Tissue engineering

* Author for correspondence. Email: justyna.drukala@uj.edu.pl, phone: +48126646145, fax: +48126646902

Abbreviations used: CME – coefficient of cell movement efficiency; PLGA – poly(L-lactide-co-glycolide); TCPS – tissue culture polystyrene; TLCD – total length of cell displacement; VCM – velocity of cell movement

INTRODUCTION

Using cultured autologous keratinocytes and fibroblasts has become a common clinical procedure in the treatment of skin defects, such as burn injury or chronic ulcers [1–3]. The advantages of this technique include high rates of engraftment and rapid wound closure. Furthermore, it can reduce requirements for the harvesting of donor skin, suppressing scar formation and reducing the need for additional reconstructive surgical procedures [4, 5]. That said, this method has a few limitations. Keratinocytes and fibroblasts are adherent cells and must be cultured on solid substrates. The trypsinization step necessary to harvest them from culture dishes breaks anchoring proteins and reduces their uptake ratio after transplantation [6–8]. Our clinical experience confirms that using fibrin sealant as a carrier of epidermal cells is very effective and improves cell uptake [9]. However, in the case of full-thickness burns, there is a need to use more profound grafts or dermal substitutes.

Advanced skin tissue engineering strategies involve the fabrication of artificial scaffolds that act as analogues of the extracellular matrix and promote cell adhesion, proliferation, migration, differentiation, and tissue regeneration in three dimensions. The most frequently used biomaterial in skin analogues is collagen I – the main component of the connective tissue matrix. Many collagen scaffolds are available on the medical market, such as Integra [10, 11], Apligraf [12], TransCyte [13] or OrCel [14]. Due to the high cost of these skin substitutes, their possible immunogenicity, and problems with processability, there is a need to find alternative biomaterials to act as cell carriers to the wound site.

Aliphatic polyesters, namely polylactides, polyglycolide, poly- ϵ -caprolactone and their copolymers, can be considered suitable materials for skin tissue engineering. The main advantage of these materials is that their degradation proceeds through the hydrolysis of their ester bonds into compounds that are naturally present in the organism, and these can be metabolized [15]. For decades, these degradable polymers have been widely used as sutures or bone fixation devices and as drug delivery systems [16, 17]. More recently, foils produced from poly(lactide-co- ϵ -caprolactone), available under the trade names of Topkin (Biomed Merck BioMaterials GmbH) or Oprafol (Lohmann & Rauscher), have been used as resorbable wound dressings [18, 19]. Skin substitutes in the form of membranes based on DL-lactide (> 70%), trimethylenecarbonate and ϵ -caprolactone (Suprathel, PolyMedics Innovations GmbH) [20] were introduced afterwards. It was found that the membranes adhered rapidly to the wound, protecting it against infections and promoting tissue healing [20, 21]. The key benefits of these products are that degradation products reduce pH at the implantation site, speeding up the healing process and providing antibacterial properties. Additionally, resorption means there is no painful removal after the wound heals completely [20–22]. Moreover, there is evidence that aliphatic polyesters are suitable materials for skin tissue engineering, as exemplified by the medical product Dermagraft, which has been commercially available since 2001.

It consists of cryopreserved neonatal fibroblasts seeded on a poly(glycolide-co-lactide) 90:10 three-dimensional mesh [23].

In previous studies, we performed *in vitro* and *in vivo* analyses of several resorbable aliphatic polyesters containing L-lactide, glycolide, ϵ -caprolactone and trimethylene carbonate with the aim of using them in bone tissue engineering [24–27]. All of our materials were synthesized with the use of a biocompatible initiator (zirconium (IV) acetylacetonate), in contrast to typically applied tin compounds [28, 29]. We wanted to reduce the risk of release of potentially harmful traces of tin compounds during the degradation of the polymers [30]. We found that the most cytocompatible material with osteoblasts, fibroblasts and macrophages *in vitro* was poly(L-lactide-co-glycolide) (PLGA) with an L-lactide to glycolide molar ratio of 85:15 [29, 31]. We also found that PLGA processed into foils and scaffolds degrades through hydrolysis and that the degradation kinetics depend on the form and microstructure of the materials. Interestingly, PLGA foils lose their integrity within 7 weeks and completely degrade within 30 weeks, as shown with *in vitro* and *in vivo* experiments [32].

In this study, we studied the possible application of PLGA processed into thin films for skin tissue engineering, with the aim of using such films as autologous cell carriers to the wound area. The application of cell lines has many limitations and might give rise to misleading conclusions due to the long cultivation time in an artificial environment. Therefore, we decided to carry out experiments on primary cells isolated directly from the human body. We analyzed the adhesion, proliferation, motility and differentiation of primary human skin keratinocytes and fibroblasts cultured on PLGA thin films for different periods of time.

MATERIALS AND METHODS

PLGA synthesis and processing into films

Copolymerization of glycolide and L-lactide (Purac) was performed in bulk with a zirconium acetylacetonate initiator, according to a previously described method [28]. To remove non-reacted monomers, the obtained copolymer was dissolved in chloroform, precipitated with cold methanol, and finally dried in a vacuum at 50°C to a constant weight.

The PLGA composition was determined using $^1\text{H-NMR}$ measurements (Varian Unity Inova spectrometer). This revealed that the molar ratio of L-lactide to glycolide was 85:15. The molecular masses were 50 kDa (M_n) and 105 kDa (M_w), as shown by gel permeation chromatography (Spectra Physics SP 8800 chromatograph). The PLGA was an amorphous polymer with a glass transition temperature (T_g) of 57.7°C, as measured by differential scanning calorimetry (DSC DuPont 1090B) [29].

The PLGA films were cast from 2% (w/v) polymer solution in methylene chloride on glass Petri dishes, followed by air drying for 24 h and vacuum drying for the next 72 h. Then, the films were detached from the Petri dishes by

6 rinses with ultra-high purity water (UHQ-water with a resistivity of 18.2 M Ω cm; Purelab UHQ). Afterwards, the films were air and vacuum dried for 24 and 72 h, respectively. The resulting films had a thickness of 20 μ m. For all of the experiments, the top surface of the films was used, i.e. the part exposed to air during the preparation process.

Surface characterization of PLGA films

The contact angles were measured by the sessile drop method using a Kruss DSA 10 Mk2 automatic drop shape analysis system. UHQ-water and diodomethane (Aldrich) droplets with a volume of 0.2 μ l were placed on each sample surface and the contact angles were obtained by averaging the results of 8 measurements for each liquid. The surface free energies were calculated from contact angles using the Owens-Wendt approach, which enables the dispersive and polar components of total surface free energy to be obtained.

Topography measurements were performed under ambient laboratory conditions with an Explorer atomic force microscope (ThermoMicroscopes, Veeco). Contact mode topographic images were recorded using Si₃N₄ probes with a spring constant of 0.05 N/m and a nominal radius of 20 nm curvature (NanoProbeTM Tips, model MLCT-EXMT-A). The images were recorded for 50 μ m x 50 μ m and 10 μ m x 10 μ m scan areas for six randomly chosen sites (300 x 300 data points) with a scan rate of 3 lines/s. All of the images were flattened using a third-order polynomial algorithm provided with the instrument. The average roughness (Ra) was calculated for all of the scanned areas.

The surface properties of reference tissue culture polystyrene (TCPS) originating from a tissue culture 6-well plate (FALCON, Becton Dickinson Labware Europe) were also evaluated. Prior to the analysis, round samples 1 cm in diameter were cut out of the bottom of the well with a hot blade. Attention was paid not to touch or contaminate the analyzed surface.

Cell culture

Human keratinocytes and fibroblasts were isolated from skin biopsies taken from three healthy donors (two females aged 32 and 67 and one male aged 48) during plastic surgery. The study was approved by the ethics committee of the Jagiellonian University, Krakow, Poland (Approval No. KBET/480/B/2003). Briefly, 0.5 cm² of each skin biopsy was washed in calcium- and magnesium-free PBS supplemented with a P/S mixture consisting of 5000 U/ml penicillin and 5 mg/ml streptomycin (all from Sigma). The rinsed skin was dissected free of fat and blood vessels and incubated in dispase (6 U/ml in PBS; Gibco) at 4°C for 16 h. After the separation of the epidermis from the dermis, the epidermis was treated with 0.05% trypsin with 2 mM EDTA (Sigma). Isolated epidermal cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Lonza) and P/S in a humidified atmosphere with 5% CO₂ at 37°C. After 24 h, the medium was changed to serum-free keratinocyte growth medium (KGM-Gold) [9].

In order to isolate fibroblasts, the dermis was cut into small pieces and incubated in collagenase I (1 mg/ml in DMEM; Sigma) at 37°C for 16 h. Cells were cultured in DMEM with 10% fetal bovine serum and P/S in a humidified atmosphere with 5% CO₂ at 37°C. Passage 1 cells were used for cell seeding.

For cell culture studies, polymeric foils were washed in 70% ethanol, sterilized with UV radiation (30 min on each side), washed in PBS and placed in culture plates. In all of the experiments, we used TCPS (FALCON, Becton Dickinson Labware Europe) as a control, except for the staining studies, for which we used glass coverslips. The cells were seeded on culture plates at a density of 5×10^3 cells/cm² for all of the experiments except the proliferative activity test (3×10^3 cells/cm²).

Cell morphology

Light microscopy studies were applied to examine the morphology of keratinocytes and fibroblasts cultured on PLGA scaffolds. Images of cell cultures were taken with a Leica Microsystems DMI-IL-LED microscope after 24, 48, 72, 96 and 120 h of cultivation.

Proliferative activity of cells

The proliferative activity of keratinocytes and fibroblasts was assessed with the aid of the Cell Proliferation Reagent WST-1 (Roche Diagnostics). After 48, 72, 96 and 120 h of cultivation, WST-1 reagent was added to each well at a 1:10 ratio to the cell culture medium. After 4 h of incubation in a humidified atmosphere with 5% CO₂ at 37°C, the plate was shaken for 1 min and 3 x 100 µl of medium from each well was transferred to a 96-well plate. The absorbance was measured at 450 nm (reference wave length 620 nm). The experiment was performed three independent times in triplicate.

Focal contact appearance and actin cytoskeleton organization

The organization of the actin cytoskeleton of the keratinocytes and fibroblasts was evaluated with F-actin staining. In order to examine focal contact formation, fibroblasts were immunostained for vinculin. Briefly, cells were fixed in 3.7% paraformaldehyde in PBS followed by incubation with PBS containing 0.1% Triton X-100 (Sigma). Fibroblasts were then blocked with 3% bovine serum albumin (Sigma) in PBS and incubated with mouse monoclonal anti-vinculin (dilution 1:200; Sigma). Goat anti-mouse IgG Alexa 488 conjugate (Invitrogen) was applied as the secondary antibody at a dilution of 1:300. Dermal cells were incubated simultaneously with a secondary antibody, TRITC-labeled phalloidin (500 ng/ml) and Hoechst 33342 dye (1 µl/ml; all from Sigma).

For the keratinocytes, after pretreatment with PBS containing 0.1% Triton X-100, the cells were incubated with TRITC-labeled phalloidin and Hoechst 33342 dye under the same conditions and solutions as the fibroblasts. Images were taken with a Leica DM-IRE-2 microscope.

Motile activity of keratinocytes

The migration of keratinocytes was examined with a Leica DMI 6000B microscope equipped with LAS AF software. The cell movement was recorded for 90 min with a time-lapse of 90 s. All of the experiments were carried out in a humidified atmosphere with 5% CO₂ at 37°C. The tracks of individual cells were determined from a series of changes in the cell centroid positions, as previously described [33], and then pooled and analyzed. The analyses were done using Hiro software v 1.0.0.4. (written by W. Czapla) to determine the velocity of cell movement (VCM, μm/min), the total length of cell displacement (TLCD, μm), and the coefficient of cell movement efficiency (CME), which corresponds to the ratio of cell displacement to cell trajectory length. The CME would equal 1 for cells moving persistently along a single straight line in a given direction and 0 for random movement. The experiment was performed three times independently. In each experiment, data were collected for 50 migrating cells.

Real-time quantitative RT-PCR analysis

In order to investigate the differentiation of keratinocytes cultured on PLGA foils, the expressions of *keratin 10* and *involucrin* were measured at the mRNA level. For both types of skin cell, analyses of *vinculin* expression at the mRNA level were carried out. Total mRNA was isolated using the RNeasy Mini Kit Plus (Qiagen GmbH) and reverse-transcribed with the High Capacity Reverse Transcription Kit (Applied Biosystems). A quantitative assessment of mRNA levels was performed via real-time RT-PCR using the 7500 Fast System instrument (Applied Biosystems) with TaqMan Gene Expression Assay including FAM-labeled probes: Hs00166289_m1 (*keratin 10*), Hs00846307_s1 (*involucrin*), Hs00419715_m1 (*vinculin*) and Hs99999905_m1 (*GAPDH*). All of the probes were from Applied Biosystems.

The reaction conditions were as follows: 95°C for 20 s; and 40 cycles at 95°C (3 s) and 60°C (30 s). The relative quantity of the target, normalized to the endogenous control *GAPDH* gene is expressed as $2^{-\Delta\Delta Ct}$ (the fold change), where: Ct is the threshold cycle; $\Delta Ct = (Ct \text{ of target genes}) - (Ct \text{ of endogenous control gene, GAPDH})$; and $\Delta\Delta Ct = (\Delta Ct \text{ of samples for target gene}) - (\Delta Ct \text{ of calibrator for the target gene})$. The experiment was performed three times independently, and all of the samples were run in duplicate.

Statistical analysis

All of the results are the means of the values obtained for cells taken from three donors, each in triplicate \pm standard deviation (SD). Data were analyzed using the nonparametric Mann-Whitney U-test. Statistical significance was defined as $p < 0.05$.

RESULTS

Surface properties of PLGA films

The values of the water and diodomethane contact angles were significantly higher for PLGA than for TCPS, while the total surface free energy and their dispersive and polar components were significantly lower (Table 1). Both substrates were transparent and smooth at the macroscopic level (data not shown), but at the nanoscale they exhibited a slightly different topography as observed under AFM (Fig. 1). Grooves 3 μm in length and up to 200 nm in

Table 1. The surface properties of TCPS and PLGA.

	Contact angle [Degree]		Surface free energy [mJ/m ²]			Roughness Ra [nm]
	Water	Diodomethane	Total	Dispersive	Polar	
TCPS	65.1 \pm 1.2	38.7 \pm 2.6	49.8 \pm 0.8	40.2 \pm 0.6	9.6 \pm 0.2	8.1 \pm 0.7
PLGA	71.4 \pm 3.6*	46.8 \pm 6.2*	43.8 \pm 1.8*	36.0 \pm 1.3*	7.8 \pm 0.5*	6.3 \pm 2.8

Mean \pm SD, n = 8 (water contact angles and surface free energy) and n = 6 (Ra roughness for scan areas of 50 μm^2 x 50 μm^2). Asterisks indicate a statistically significant difference ($p < 0.05$).

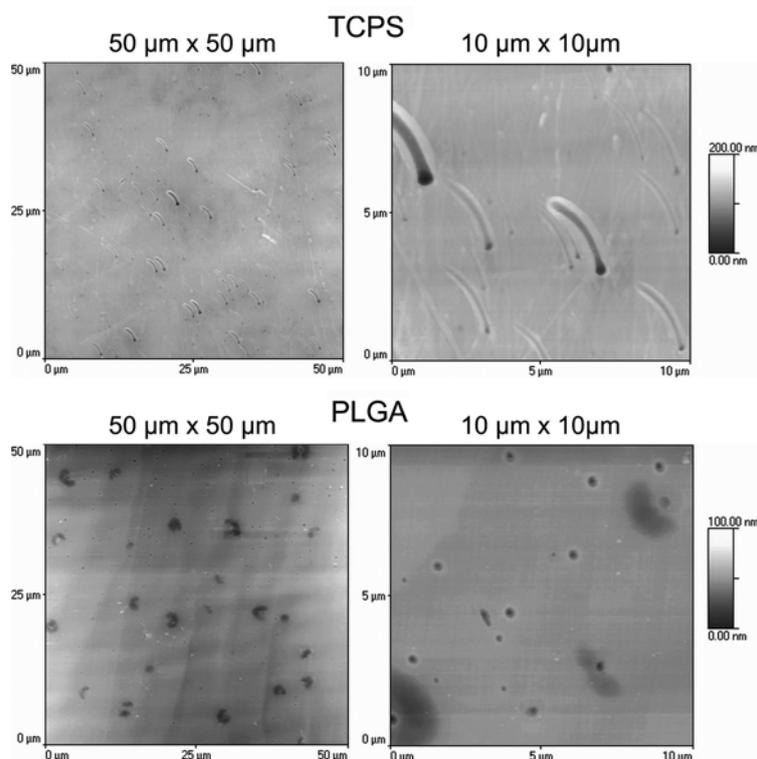


Fig. 1. AFM topographic images of the reference TCPS and PLGA films. Scan areas 50 μm x 50 μm and 10 μm x 10 μm ; z range 200 nm for TCPS and 100 nm for PLGA film.

depth were observed on TCPS. On the other hand, circular holes with a depth of 100 nm were visible on PLGA film. Circular or elongated pits 2–4 μm in size were present around some of the holes. However, there was no significant difference in the Ra roughness for TCPS and PLGA substrates. The topographical features observed on PLGA film were created during the manufacturing process, i.e. evaporation of the solvent [29]. On the other hand, the topography of TCPS was a result of injection molding. In a recent study by Zeiger et al., it was shown that both the topography and the surface chemistry of different TCPS dishes vary depending on commercial source [34]. In all of the TCPS samples scratches, fiber-like or pit-like features were detected. These are a result of injection into machined metallic molds [34]. The R_{RMS} roughness of the TCPS surfaces was found to be in the range of 1–6 nm, depending on the manufacturer, i.e. similar to the value obtained in this study. In our previous study, we found that TCPS provided by another manufacturer (Nunc) had a scratched topography with an R_{RMS} of 7 nm [35]. In summary, we found that the PLGA films were more hydrophobic and possessed lower surface free energy than TCPS. Both materials had a slightly different topography at the nanoscale, but their roughness parameter Ra was similar.

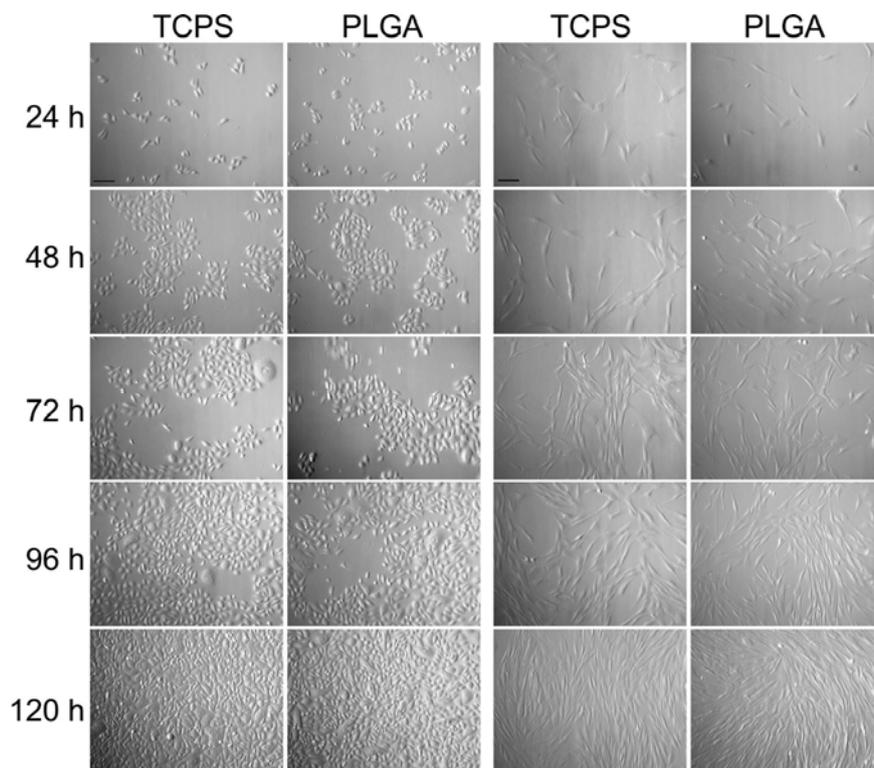


Fig. 2. Morphology of keratinocytes (left panel) and fibroblasts (right panel) on PLGA foils and TCPS after 24, 48, 72, 96, 120 h of cultivation. The pictures were taken with an optical microscope. Magnification 100x, scale bar = 100 μm .

Cell morphology and proliferative activity

Under control conditions, the skin keratinocytes exhibited typical epithelial morphology. They formed more or less regular colonies with individual migrating cells characterized by rear–front polarization and a lamellipodium at the leading edge of the cell (Fig. 2, left panel). Fibroblasts cultured on control material were elongated and after reaching confluence were organized in parallel-orientated spindle-shaped cells (Fig. 2, right panel). Variation in the morphology of epithelial and dermal cells cultured on PLGA vs. TCPS films was not visible. The proliferative activity test confirmed the microscopic observations. Differences in proliferation values between the fibroblasts cultured on PLGA films and control material (Fig. 3A) were not statistically significant. The rate of keratinocyte proliferation was comparable to the control (Fig. 3B).

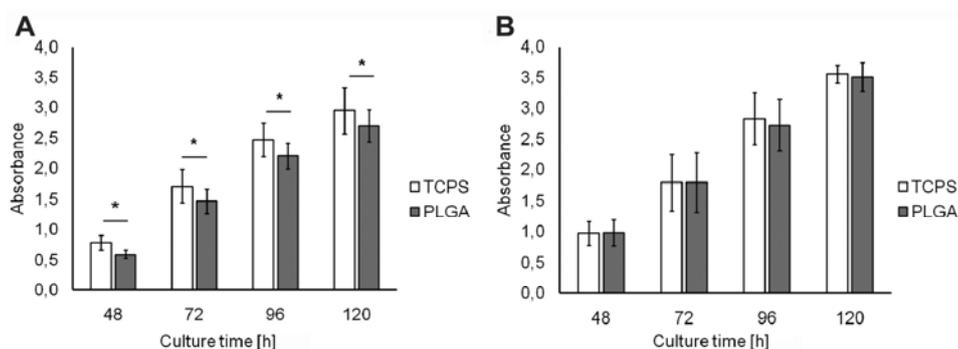


Fig. 3. The proliferative activity of keratinocytes (A) and fibroblasts (B) was examined using the Cell Proliferation Reagent WST-1 assay. The presented data are the means \pm SD. Asterisks indicate a statistically significant difference ($p < 0.05$).

Focal contact appearance, vinculin expression and actin cytoskeleton organization

One of the most important aspects of evaluating the in vitro biocompatibility of biomaterials is assessing the adherence of cells. Adherence is made possible by structures termed focal contacts, for which vinculin is a key protein. Our research revealed that the mRNA level of *vinculin* was comparable in keratinocytes and fibroblasts cultured on PLGA foils and TCPS (Fig. 4A and B). Furthermore, these results were confirmed by immunofluorescence studies carried out on fibroblasts, which are typical focal contact-forming cells (Fig. 4C, D and E).

Keratinocytes and fibroblasts have a very well-developed actin cytoskeleton. Thus, it is noteworthy that we did not observe any variation in the organization of the actin cytoskeleton of cells cultured on PLGA scaffolds and TCPS (Fig. 4D and E, Fig. 5).

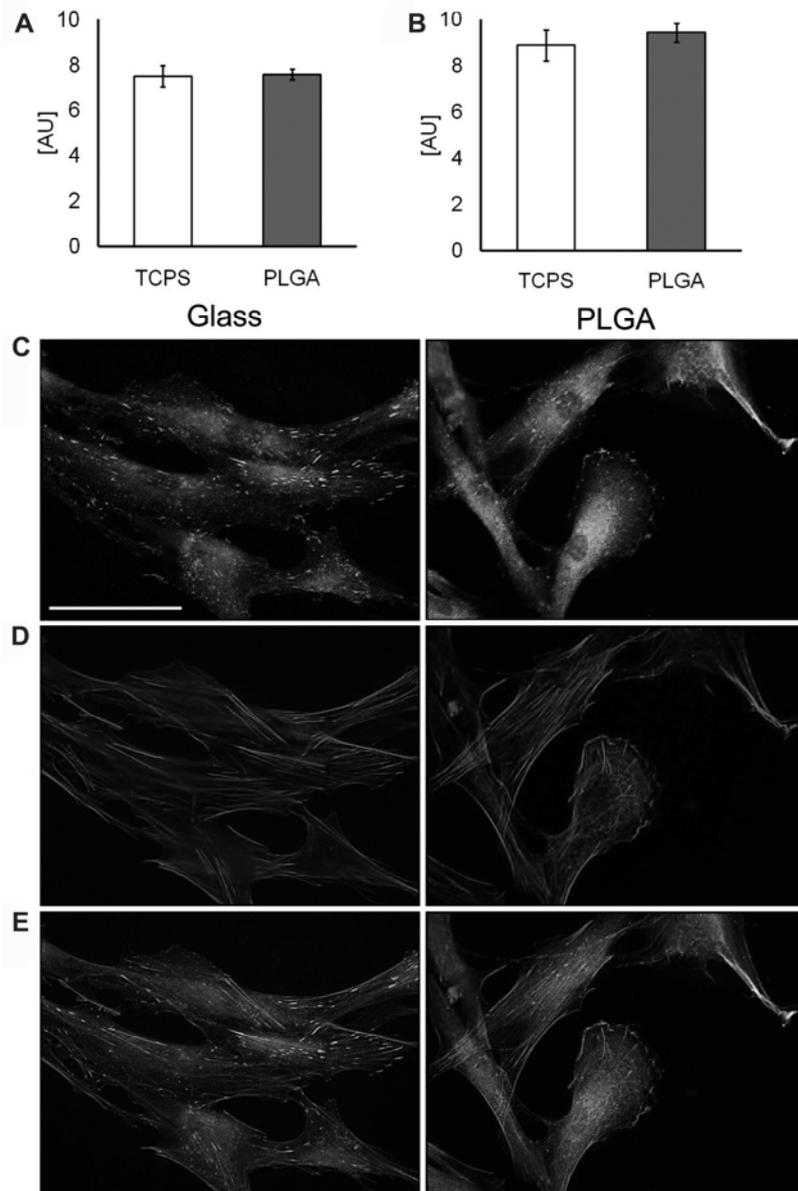


Fig. 4. The relative expression of *vinculin* in keratinocytes (A) and fibroblasts (B) was evaluated using quantitative real-time RT-PCR analyses. The presented data are the means \pm SD of three independent experiments. There were no statistically significant differences ($p < 0.05$). AU – arbitrary unit. The organization of the actin cytoskeleton of the fibroblasts was evaluated with TRITC-labeled phalloidin (F-actin staining) and the appearance of focal contacts was assessed with immunofluorescence staining of vinculin. Hoechst 33342 dye was used for DNA staining. Vinculin (C); F-actin (D); vinculin, F-actin and nuclear staining (E). Magnification 400x, scale bar = 100 μ m.

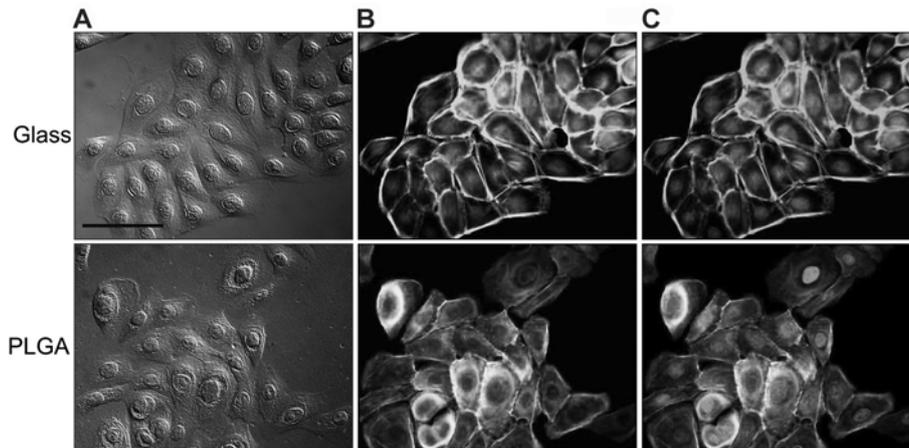


Fig. 5. The organization of the actin cytoskeleton of keratinocytes was evaluated with TRITC-labeled phalloidin (F-actin staining). Hoechst 33342 dye was used for DNA staining. A – Cells in the bright field. B – F-actin. C – F-actin and nuclear staining. Magnification 400x, scale bar = 100 μm .

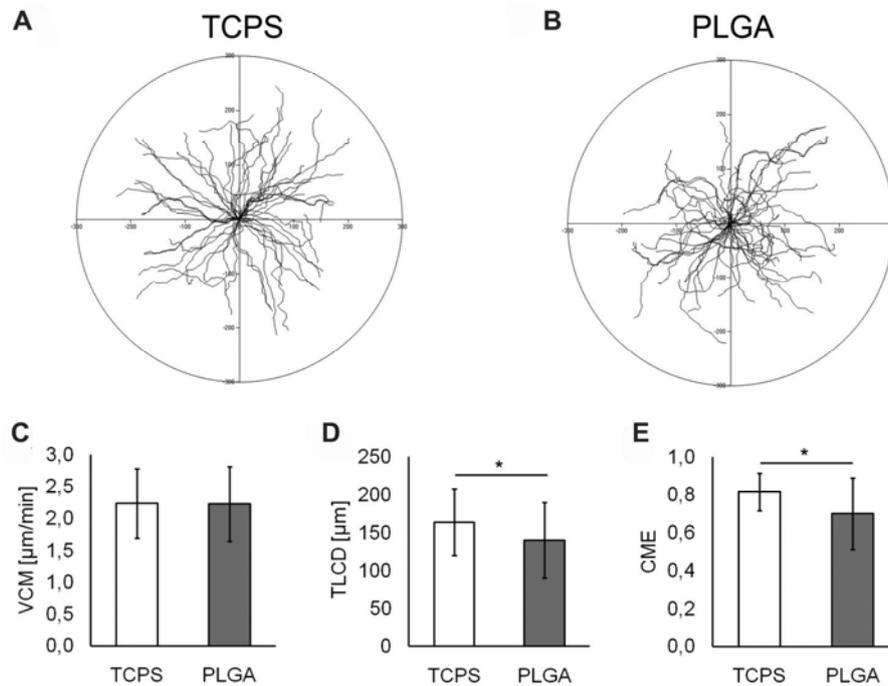


Fig. 6. The trajectories of 50 migrating cells cultured on TCPS (A) and PLGA foils (B) are shown in the form of circular diagrams (axis scale in μm). The presented data are the means \pm SD from velocity of cell movement (C), total length of cell displacement (D) and coefficient of cell movement efficiency (E) measurements for 150 cells, from three independent experiments. Asterisks indicate a statistically significant difference ($p < 0.05$).

Epidermal cell motility

Keratinocyte migration is important for effective and rapid wound closure. We analyzed the two-dimensional epidermal cell locomotion using time-lapse monitoring of cell movement. We observed that keratinocyte motility on PLGA films was comparable to the control material, with no statistically significant differences in velocity of cell movement ($2.23 \pm 0.54 \mu\text{m}/\text{min}$ vs. $2.23 \pm 0.59 \mu\text{m}/\text{min}$; $p < 0.05$; Fig. 6C). The circular graphs (Fig. 6A and B) show that cells on PLGA films twisted more frequently. This is confirmed by a decrease in the total length of cell displacement ($164 \pm 44 \mu\text{m}$ vs. $140 \pm 50 \mu\text{m}$; $p < 0.05$; Fig. 6D), and consequently, less efficient cell migration (CME 0.82 ± 0.10 vs. 0.70 ± 0.19 ; $p < 0.05$; Fig. 6E).

Keratinocyte differentiation

An ideal scaffold for skin tissue engineering should promote not only the proliferation of epidermal cells, but also their proper differentiation. In further experiments, we examined the relative expression of post-mitotic *keratin 10* (early differentiation, Fig. 7A) and *involucrin*, a component of the cornified envelope (terminal differentiation, Fig. 7B). The differences in the relative expressions of these genes when cells were cultured on PLGA foils and TCPS were not statistically significant.

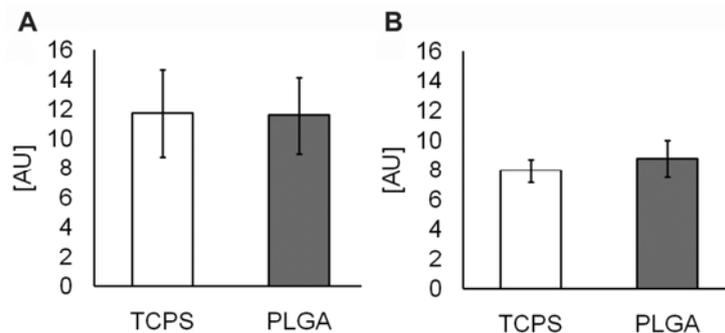


Fig. 7. The relative expressions of markers of keratinocyte differentiation. Real-time RT-PCR analyses were performed to evaluate the gene expression of post-mitotic *keratin 10* (early differentiation, panel A) and the component of the cornified envelope, *involucrin* (terminal differentiation, panel B). The presented data are the means \pm SD of three independent experiments. There were no statistically significant differences ($p < 0.05$). AU – arbitrary unit.

DISCUSSION

The main goal of tissue engineering is the *in vitro* reconstruction of tissue to accelerate the regeneration process. Skin tissue engineering requires the application of autologous cell carriers to the wound area in order to provide the appropriate spatial organization of the tissue.

One currently investigated biomaterial is thin films made of the resorbable polymer PLGA, which biodegrades after fulfilling its biological function (i.e. the

delivery of autologous cells and wound healing) and therefore does not require painful removal. The aim of this study was to examine if PLGA films influence some particular functions of human skin cells cultured on them for different periods of time. TCPS, regarded as the best material for cell culture, was applied as a reference.

The biocompatibility of PLGA was widely investigated in earlier research. For example, the studies of Kumbar et al. (2008) showed that cells on electrospun PLGA matrices had well spread morphology and progressive growth [36]. However, when compared to TCPS, the proliferation ratio of fibroblasts cultured on these scaffolds was significantly decreased [36]. Studies performed previously by our group showed that PLGA processed into form foils stimulated the typical functions of L929 fibroblasts [26, 27, 37] and did not induce the release of proinflammatory cytokines from macrophages [29]. Moreover, PLGA foils were found to degrade almost completely after 30 weeks of implantation into rat skeletal muscle [32].

Furthermore, Garric et al. found that PLGA films stimulated fibroblast proliferation but inhibited keratinocyte proliferation [38]. Our results indicate an absence of significant differences in the proliferation ratios of fibroblasts and keratinocytes cultured on PLGA thin films when compared to TCPS (Fig. 3A and B).

Our studies have also shown that PLGA thin films do not affect the basic functions of primary human keratinocytes and fibroblasts, such as morphology (Fig. 2), proliferation (Fig. 3), and actin cytoskeleton organization and appearance of focal contacts (Figs 4 and 5). Moreover, the migration of keratinocytes, critical for rapid wound closure, was comparable when cells were cultured on PLGA foils and TCPS (Fig. 6).

We only found differences in the trajectories of migrating cells. Keratinocytes cultured on PLGA tend to turn more frequently. As a result we observed statistically significant differences in the values of the total length of cell displacement and the coefficient of cell movement efficiency when compared to TCPS. It can be attributed to different topography as shown by AFM (Fig. 1), or to the higher hydrophobicity and lower surface free energy of PLGA (Tab. 1). However, recent studies of Zeiger et al., who studied TCPS produced by different manufacturers, showed that final surface topography more strongly dictated cell response than surface wettability, because topographic features were able directly govern the extension of cell filopodia [34]. Such cell type-specific responses to the underlying substrate were reported for fibroblasts [34], endothelial cells, smooth muscle cells [39] and nerve cells [40].

Another important phase of wound healing is the formation of stratified epithelium with appropriate differentiation of keratinocytes. We decided to check if PLGA foils influence this process. It is well known that the state of differentiation of epidermal keratinocytes is reflected by the intricate expression pattern of keratins, which form the intermediate filament cytoskeleton [41, 42]. Herein, we examined the mRNA expression level of *keratin 10*, which is engaged in the early differentiation stage, and *involucrin*, a component of the

cross-linked envelope [43]. We did not observe any disturbances in the differentiation process when PLGA films were used (Fig. 7A and B).

Experiments conducted by Blackwood et al. (2008) showed significant contraction of PLGA material after cell seeding [44]. Interestingly, we did not notice any contraction in our tests, suggesting that our PLGA films are promising material for cell carriers from the point of view of their biofunctionality.

Our results indicate that PLGA films do not affect the basic functions of primary human skin keratinocytes and fibroblasts related to tissue regeneration, such as adherence, proliferation, migration and differentiation. Thus, PLGA thin films show acceptable biocompatibility *in vitro*, paving the way for their applications as biomaterials for skin tissue engineering.

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Conflict of interest. The authors declare no conflict of interest.

REFERENCES

1. Bannasch, H., Fohn, M., Unterberg, T., Bach, A.D., Weyand, B. and Stark, G.B. Skin tissue engineering. *Clin. Plast. Surg.* **30** (2003) 573–579. DOI: 10.1016/S0094-1298(03)00075-0.
2. Bello, Y.M., Falabella, A.F. and Eaglstein, W.H. Tissue-engineered skin. Current status in wound healing. *Am. J. Clin. Dermatol.* **2** (2001) 305–313. DOI: 10.2165/00128071-200102050-00005.
3. Kirsner, R.S., Falanga, V. and Eaglstein, W.H. The development of bioengineered skin. *Trends Biotechnol.* **16** (1998) 246–249. DOI: 10.1016/S0167-7799(98)01196-2.
4. Boyce, S.T., Goretsku, M.J., Greenhalgh, D.G., Kagan, R.J., Rieman, M.T. and Warden, G.D. Comparative assessment of cultured skin substitutes and native skin autograft for treatment of full-thickness burns. *Ann. Surg.* **222** (1995) 743–752. DOI: 10.1097/00000658-199512000-00008.
5. Boyce, S.T., Kagan, R.J., Yakuboff, K.P., Meyer, N.A., Rieman, M.T., Greenhalgh, D.G. and Warden, G.D. Cultured skin substitutes reduce donor

- skin harvesting for closure of excised, full-thickness burns. **Ann. Surg.** 235 (2002) 269–279. DOI: 10.1097/0000658-200202000-00016.
6. Voigt, M., Schauer, M., Schaefer, D.J., Andree, C., Horch, R. and Stark, G.B. Cultured epidermal keratinocytes on a microspherical transport system are feasible to reconstitute the epidermis in full-thickness wounds. **Tissue Eng.** 5 (1999) 563–572. DOI: 10.1089/ten.1999.5.563.
 7. Woodley, D.T., Peterson, H.D., Herzog, S.R., Stricklin, G.P., Burgeson, R.E., Briggaman, R.A., Cronce, D.J. and O’Keefe, E.J. Burn wounds resurfaced by cultured epidermal autografts show abnormal reconstitution of anchoring fibrils. **JAMA** 259 (1988) 2566–2571. DOI: 10.1001/jama.1988.03720170042031.
 8. Herndon, D.N. and Rutan, R.L. Comparison of cultured epidermal autograft and massive excision with serial autografting plus homograft overlay. **J. Burn Care Rehabil.** 13 (1992) 154–157. DOI: 10.1097/00004630-199201000-00034.
 9. Drukala, J., Bandura, L., Cieslik, K. and Korohoda, W. Locomotion of human skin keratinocytes on polystyrene, fibrin, and collagen substrata and its modification by cell-to-cell contacts. **Cell Transplant.** 10 (2001) 765–771. DOI: 10.3727/000000001783986251.
 10. Yannas, I.V., Burke, J.F. and Gordon, P.L. Design of an artificial skin II. Control of chemical composition. **J. Biomed. Mater. Res.** 14 (1980) 107–131. DOI: 10.1002/jbm.820140203.
 11. Heimbach, D., Luterman, A. and Burke, J.F. Artificial dermis for major burns; a multi-center randomized clinical trial. **Ann. Surg.** 208 (1988) 313–320. DOI: 10.1097/0000658-198809000-00008.
 12. Eaglstein, W.H., Iriondo, M. and Laszlo, K. A composite skin substitute (Graftskin) for surgical wounds: a clinical experience. **Dermatol. Surg.** 21 (1995) 839–843. DOI: 10.1111/j.1524-4725.1995.tb00709.x.
 13. Noordenbos, J., Dore, C. and Hansbrough, J.F. Safety and efficacy of TransCyte for the treatment of partial-thickness burns. **J. Burn Care Rehabil.** 20 (1999) 275–281. DOI: 10.1097/00004630-199907000-00002.
 14. Stephens, R., Wilson, K. and Silverstein, P. A premature infant with skin injury successfully treated with bilayered cell matrix. **Ostomy/Wound Manage** 48 (2002) 34–38.
 15. Gopferich, A. Mechanisms of polymer degradation and erosion. **Biomaterials** 17 (1996) 103–114. DOI: 10.1016/0142-9612(96)85755-3.
 16. Ignatius, A.A. and Claes, L.E. In vitro biocompatibility of bioresorbable polymers: poly(L, DL-lactide) and poly(L-lactide-co-glycolide). **Biomaterials** 17 (1996) 831–839. DOI: 10.1016/0142-9612(96)81421-9.
 17. Mendes, S.C., Reis, R.L., Bovell, Y.P., Cunha, A.M., van Blitterswijk, C.A. and de Bruijn, J.D. Biocompatibility testing of novel starch-based materials with potential application in orthopaedic surgery: a preliminary study. **Biomaterials** 22 (2001) 2057–2064. DOI: 10.1016/S0142-9612(00)00395-1.

18. Porte, T., Faschingbauer, M., Seide, K. and Jurgens, C. Topkin – eine resorbierbare folie aus lactid-caprolacton. **Trauma Berufskrankh.** 5 (2003) 443–448. DOI: 10.1007/s10039-003-0811-3.
19. Jurgens, C., Schulz, A.P., Porte, T., Faschingbauer, M. and Seide, K. Biodegradable films in trauma and orthopedic surgery. **Eur. J. Trauma** 32 (2006) 160–171. DOI: 10.1007/s00068-006-6051-z.
20. Uhlig, C., Rapp, M., Hartmann, B., Hierlemann, H., Planck, H. and Dittel, K.K. Suprathel – an innovative, resorbable skin substitute for the treatment of burn victims. **Burns** 33 (2007) 221–229. DOI: 10.1016/j.burns.2006.04.024.
21. Selig, H.F., Keck, M., Lumenta, D.B., Mittlbock, M. and Kamolz, L.P. The use of a polylactide-based copolymer as a temporary skin substitute in deep dermal burns: 1-year follow-up results of a prospective clinical noninferiority trial. **Wound Repair Regen.** 21 (2013) 402–409. DOI: 10.1111/wrr.12050.
22. Keck, M., Selig, H.F., Lumenta, D.B., Kamolz, L.P., Mittlbock, M. and Frey, M. The use of Suprathel in deep dermal burns: first results of a prospective study. **Burns** 38 (2012) 388–395. DOI: 10.1016/j.burns.2011.09.026.
23. Lysaght, M.J. and Hazlehurst, A.L. Tissue engineering: The end of the beginning. **Tissue Eng.** 10 (2004) 309–320. DOI: 10.1089/107632704322791943.
24. Pamula, E., Bacakova, L., Filova, E., Buczynska, J., Dobrzynski, P., Noskova, L. and Grausova, L. The influence of pore size on colonization of poly(L-lactide-glycolide) scaffolds with human osteoblast-like MG 63 cells in vitro. **J. Mater. Sci. Mater. Med.** 19 (2008) 425–435. DOI: 10.1007/s10856-007-3001-1.
25. Pamula, E., Filova, E., Bacakova, L., Lisa, V. and Adamczyk, D. Resorbable polymeric scaffolds for bone tissue engineering: the influence of their microstructure on the growth of human osteoblast-like MG 63 cells. **J. Biomed. Mater. Res. A** 89 (2009) 432–443. DOI: 10.1002/jbm.a.31977.
26. Scislowska-Czarnecka, A., Pamula, E. and Kolaczowska, E. Impact of poly(L-lactide) versus poly(L-lactide-co-trimethylene carbonate) on biological characteristics of fibroblasts and osteoblasts. **Folia Biol.** 61 (2013) 11–24. DOI: 10.3409/fb61_1-2.11.
27. Scislowska-Czarnecka, A., Pamula, E. and Kolaczowska, E. Biocompatibility evaluation of glycolide-containing polyesters in contact with osteoblasts and fibroblasts. **J. Apply. Polymer Sci.** 127 (2013) 3256–3268. DOI: 10.1002/app.37762.
28. Dobrzynski, P., Kasperczyk, J., Janeczek, H. and Bero, M. Synthesis of biodegradable copolymers with the use of low toxic Zirconium compounds. 1. Copolymerization of glycolide with L-lactide initiated by Zr(Acac)₄. **Macromolecules** 34 (2001) 5090–5098. DOI: 10.1021/ma0018143.
29. Pamula, E., Dobrzynski, P., Szot, B., Kretek, M., Krawciow, J., Plytycz, B. and Chadzinska, M. Cytocompatibility of aliphatic polyesters – in vitro study on fibroblasts and macrophages. **J. Biomed. Mater. Res. A** 87 (2008) 524–535. DOI: 10.1002/jbm.a.31802.

30. Tanzi, M.C., Verderio, M.G., Lampugnani, M.G., Resnati, M., Dejana, E. and Sturani E. Cytotoxicity of some catalysts commonly used in the synthesis of copolymers for biomedical use. **J. Mater. Sci.** 5 (1994) 393–396. DOI: 10.1007/BF00058971.
31. Douglas, T., Pamula, E., Hauk, D., Wiltfang, J., Sivananthan, S., Sherry, E. and Warnke, P.H. Porous polymer/hydroxyapatite scaffolds: characterization and biocompatibility investigations. **J. Mater. Sci. Mater. Med.** 20 (2009) 1909–1915. DOI: 10.1007/s10856-009-3756-7.
32. Pamula, E. and Menaszek, E. In vitro and in vivo degradation of poly(L-lactide-co-glycolide) films and scaffolds. **J. Mater. Sci. Mater. Med.** 19 (2008) 2063–2070. DOI: 10.1007/s10856-007-3292-2.
33. Miekus, K. and Madeja Z. Genistein inhibits the contact-stimulated migration of prostate cancer cells. **Cell. Mol. Biol. Lett.** 12 (2007) 348–361. DOI: 10.2478/s11658-007-0007-0.
34. Zeiger, A.S., Hinton, B. and Van Vliet, K.J. Why the dish makes a difference: Quantitative comparison of polystyrene culture surfaces. **Acta Biomater.** 9 (2013) 7354–7361. DOI: 10.1016/j.actbio.2013.02.035.
35. Adamczak, M., Scisłowska-Czarnecka, A., Genet, M.J., Dupont-Gillain C.C. and Pamula E. Surface characterization, collagen adsorption and cell behaviour on poly(L-lactide-co-glycolide). **Acta Bioeng. Biomech.** 13 (2011) 63–75.
36. Kumbar, S.G., Nukavarapu, S.P., James, R., Nair, L.S. and Laurencin, C.T. Electrospun poly(lactic acid-co-glycolic acid) scaffolds for skin tissue engineering. **Biomaterials** 29 (2008) 4100–4107. DOI: 10.1016/j.biomaterials.2008.06.028.
37. Scisłowska-Czarnecka, A., Pamula, E., Tlalka, A. and Kolaczkowska, E. Effects of aliphatic polyesters on activation of the immune system: Studies on macrophages. **J. Biomater. Sci. Polymer Ed.** 23 (2012) 715–738. DOI: 10.1163/092050611X559421.
38. Garric, X., Moles, J.P., Garreau, H., Braud, C., Guilhou, J.J. and Vert, M. Growth of various cell types in the presence of lactic and glycolic acids: the adverse effect of glycolic acid released from PLAGA copolymer on keratinocyte proliferation. **J. Biomater. Sci. Polymer Ed.** 13 (2002) 1189–1201. DOI: 10.1163/156856202320892957.
39. Biela, S.A., Su, Y., Spatz, J.P. and Kemkemer R. Different sensitivity of human endothelial cells, smooth muscle cells and fibroblasts to topography in the nano-micro range. **Acta Biomater.** 5 (2009) 2460–2466. DOI: 10.1016/j.actbio.2009.04.003.
40. Hoffman-Kim D., Mitchel J.A. and Bellamkonda R.V. Topography, cell response, and nerve regeneration. **Annu. Rev. Biomed. Eng.** 12 (2010) 203–231. DOI: 10.1146/annurev-bioeng-070909-105351.
41. Fuchs, E. and Green, H. Changes in keratin gene expression during terminal differentiation of the keratinocyte. **Cell** 19 (1980) 1033–1042. DOI: 10.1016/0092-8674(80)90094-X.

42. Moll, R., Franke, W.W., Schiller, D.L., Geiger, B. and Krepler, R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. **Cell** 31 (1982) 11–24. DOI: 10.1016/0092-8674(82)90400-7.
43. Watt, F. Involucrin and other markers of keratinocyte terminal differentiation. **J. Invest. Dermatol.** 81 (1983) 100–103. DOI: 10.1111/1523-1747.ep12540786.
44. Blackwood, K.A., McKean, R., Canton, I., Freeman, C.O., Franklin, K.L., Cole, D., Brook, I., Farthing, P., Rimmer, S., Haycock, J.W., Ryan, A.J. and MacNeil, S. Development of biodegradable electrospun scaffolds for dermal replacement. **Biomaterials** 29 (2008) 3091–3104. DOI: 10.1016/j.biomaterials.2008.03.037.