Chapter

Nanofibrous Scaffolds for Skin Tissue Engineering and Wound Healing Based on Synthetic Polymers

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Abstract

Nanofibrous scaffolds are popular materials in all areas of tissue engineering, because they mimic the fibrous component of the natural extracellular matrix. In this chapter, we focused on the application of nanofibers in skin tissue engineering and wound healing, because the skin is an organ with several vitally important functions, particularly barrier, thermoregulatory, and sensory functions. Nanofibrous meshes not only serve as carriers for skin cells but also can prevent the penetration of microbes into wounds and can keep appropriate moisture in the damaged skin. The nanofibrous meshes have been prepared from a wide range of synthetic and nature-derived polymers. This review is concentrated on synthetic non-degradable and degradable polymers, which have been explored for skin tissue engineering and wound healing. These synthetic polymers were often combined with natural polymers of the protein or polysaccharide nature, which improved their attractiveness for cell colonization. The nanofibrous scaffolds can also be loaded with various bioactive molecules, such as growth factors, hormones, vitamins, antioxidants, antimicrobial, and antitumor agents. In advanced tissue engineering approaches, the cells on the nanofibrous scaffolds are cultured in dynamic bioreactors enabling appropriate mechanical stimulation of cells and at air-liquid interface. This chapter summarizes recent results achieved in the field of nanofiber-based skin tissue engineering, including results of our research group.

Keywords: skin replacements, wound dressings, nanofibers, electrospinning, epidermis, dermis, keratinocytes, fibroblasts, stem cells, vascularization, cell delivery, drug delivery, regenerative medicine

1. Introduction

The skin is the largest organ of the human body with several vitally important functions. The skin acts as barrier against adverse effects of the surrounding

environment on the organism, such as chemical factors, radiation factors, particularly ultraviolet light, and microbial infection. Other important functions of skin include thermoregulation, sensation of temperature, touch, pressure, and pain, keeping appropriate moisture in the underlying tissues, excretion of ions, water, and various biomolecules (e.g., lipids and proteins), and also production and storage of various biomolecules, such as pigments, vitamin D, and keratins for formation of epidermal appendages (for a review, see [1, 2]). Skin severely and chronically damaged by trauma, burns, bedsores, and by various diseases, e.g., diabetes, cannot exert these functions, which can lead to amputation and even death. Therefore, there is essential need to regenerate the damaged skin, particularly by methods of skin tissue engineering and induction of active wound healing. For these purposes, nanofibrous scaffolds seem to be one of the most promising materials. Nanomaterials in general are defined as features not exceeding 100 nm at least in one dimension, i.e., in diameter in case of nanofibers. However, nanofibers usually used in tissue engineering are often thicker (i.e., several hundreds of nm). In fact, they are submicron-scale fibers, but the term "nanofibers" has become widely used for them. Nanofibers can be obtained by various techniques, such as biological synthesis (e.g., nanocellulose produced by bacteria), self-assembly, phase separation, interfacial polymerization, suitable for electrically conductive materials, melt processing or antisolvent precipitation, and particularly by electrospinning, which has emerged as a relatively simple, elegant, scalable, and efficient technique for fabrication of polymeric nanofibers (for a review, see [2–5]).

The advantage of nanofibrous scaffolds is that they mimic the fibrous component of the natural extracellular matrix (ECM), and therefore they can serve as ECM analogues for tissue engineering. In addition, nanofibrous meshes can act as a protective barrier against penetration of microbes into wounds, can keep the moisture in the damaged skin, and, at the same time, allow gas exchange and absorb the exudate from the wounds. These meshes can also be loaded with various bioactive molecules, such as growth and angiogenic factors, cytokines, hormones, vitamins, antioxidants, antimicrobial and antitumor agents, amino acids (L-arginine), wound healing peptides (e.g., melanocyte-inhibiting factor), and with antimicrobial peptides [6–8]; for a review, see [1, 2]. Therefore, nanofibers can serve not only as tissue engineering scaffolds for skin cells but also as carriers for controlled drug delivery into skin.

The nanofibrous scaffolds for skin tissue engineering and wound healing have been prepared from a wide range of synthetic and nature-derived polymers. Both these groups of polymers contain polymers relatively easily degradable in the human organism, and polymers which are non-degradable or slowly degradable. This review is focused on synthetic polymers, which have been used for creation of nanofibrous scaffolds for skin tissue engineering and wound healing applications. Typical and widely used degradable synthetic polymers include polylactides [9, 10] and their copolymers with polyglycolides [11], or polycaprolactone [6, 12] and its copolymers with polylactides [13]. Examples of biostable synthetic polymers are polyurethane [7], polydimethylsiloxane [14], polyethylene terephthalate [15] or polyethersulfone [16]. Polymers degradable in the human body are suitable as direct scaffolds for skin tissue engineering, while biostable polymers can be rather recommended as "intelligent" wound dressings delivering cells (keratinocytes, fibroblasts or stem cells) and bioactive molecules into wounds.

However, polymers in nanofibrous scaffolds are predominantly used in various combinations—synthetic with natural, degradable with non-degradable—and also in combination with various nanoparticles, e.g., mineral nanoparticles [17], carbon-based nanoparticles [1, 18] or metal-based nanoparticles [19, 20]. The reason of these combinations is to improve the stability, spinnability, wettability, mechanical

properties, and bioactivity of nanofibers. The combination of various polymers, nanoparticles, and other components is a strategy commonly used to obtain hybrid materials possessing properties better than those of the individual constituents, regarding their use in scaffolds for tissue engineering or as material for wound dressing [21]. For example, synthetic polymers do not contain adhesion motifs recognizable by cell adhesion receptors, and combination with nature-derived polymers, which are proteins (collagen, gelatin, keratin, fibrin [6, 10, 22, 23]) or polysaccharides (hyaluronic acid, sulfated glycosaminoglycans, such as heparin [24, 25]) can endow them with these motifs, because these polymers are often components of ECM.

In electrospun nanofibrous meshes, the polymers can be combined by various approaches. In blending electrospinning, the polymers are mixed, filled in the same syringe, and electrospun together, which results in creation of fibers with two or more components randomly distributed within a fiber. In multi-jet electrospinning, each polymer is filled in a separate syringe and electrospun individually, which results in creation of meshes with two or more types of nanofibers. These types of nanofibers can be electrospun either concurrently and distributed randomly (i.e., mixing electrospinning) or alternatively and arranged into separate layers (i.e., multilayering electrospinning). In co-axial electrospinning, hybrid nanofibers with a core-shell architecture are created by spinning of two different solutions filled into outer and inner compartments of a co-axial syringe. Finally, an electrospun polymer can be secondarily coated with other polymers or bioactive substances [22, 26–28] (**Figure 1**).

Nanofibrous meshes for skin tissue engineering can also be combined with other material types, such as porous 3D scaffolds or hydrogels in order to reconstruct two main skin layers, i.e., epidermis containing keratinocytes and dermis containing fibroblasts [23, 29–31]. Another advanced approach promising for construction of dermo-epidermal replacements is centrifugal jet spinning, capable of large-scale production of nanofibrous 3D scaffolds [32, 33].

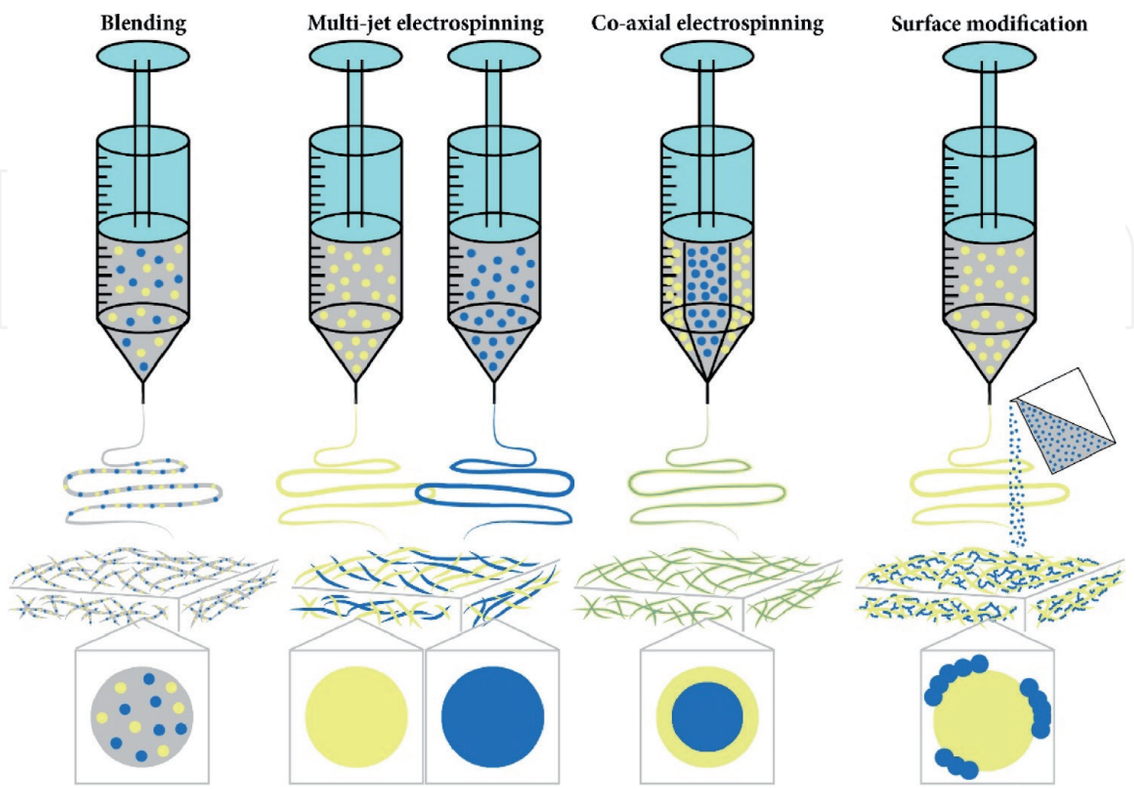

Figure 1.
Modes of combination of various polymers and compounds in nanofibrous scaffolds.

In the waste majority of studies dealing with skin tissue engineering based on nanofibrous scaffolds, keratinocytes have been cultivated in a conventional static cell culture system, submerged into the culture media, although under physiological condition *in vivo*, keratinocytes are exposed to air. Therefore, in advanced skin tissue engineering, it is necessary to cultivate keratinocytes under appropriate mechanical loading, i.e., strain stress [34] or pressure stress [35], and simultaneously to cultivate them on the scaffolds exposed to the air-liquid interface [34, 36].

This chapter summarizes earlier and recent knowledge on skin tissue engineering and wound dressing applications, based on nanofibrous scaffolds made of synthetic non-degradable and degradable polymers, including our results.

2. Nanofibers from synthetic non-degradable polymers

Synthetic non-degradable polymers, explored for creation of electrospun nanofibrous meshes for skin regenerative therapies, included polyurethane, polydimethylsiloxane, polyethylene terephthalate, polyethersulfone, and even polystyrene. This group of polymers also includes non-degradable hydrogels, such as poly(acrylic acid), poly(methyl methacrylate), and poly(di(ethylene glycol) methyl ether methacrylate).

Polyurethane (PU) has been most frequently used from the mentioned polymers, which is due to its elasticity, and also possibility to prepare it in a degradable form, e.g., as poly(ester-urethane) urea (PEUU), which facilitates its applicability in skin tissue engineering [37], while the non-degradable forms of PU (and other non-degradable polymers in general) are rather used in wound dressing applications. Non-degradable PU nanofibrous meshes has been tested as advanced wound dressings loaded with various healing, angiogenic, anti-inflammatory, antioxidant, and antimicrobial substances. For example, blending PU with propolis improved the mechanical strength and hydrophilicity of the nanofibrous membrane, its cytocompatibility with fibroblasts and its antibacterial activity [38]. Blending PU with virgin olive oil endowed the nanofibrous meshes with photoprotective and antioxidant properties [19]. Dextran in composite PU/dextran fibers had angiogenic activity, and also served as a carrier for incorporation of β-estradiol, which accelerated healing of acute cutaneous wounds by its potent anti-inflammatory activity [7]. Another promising nanofibrous membrane applicable for wound dressing was prepared from electrospun PU, treated by plasma and subsequent spraying with chitosan solution containing an inclusion complex of β-cyclodextrin encapsulating berberine, i.e., an isoquinoline alkaloid with antimicrobial and anti-inflammatory activity [39]. Other antimicrobial substances incorporated into PU-based nanofibers included silver nanoparticles [20, 40], copper oxide nanocrystals [19] and antibiotics, such as silver-sulfadiazine [41] and amoxicillin [37]. In general, all these scaffolds showed none or low toxicity towards human HaCaT keratinocytes [20] or fibroblasts [19, 40], and no adverse reactions when implanted into laboratory animals *in vivo* [37, 41]. In addition, copper oxide is known by its angiogenic activity [19]. Nanofibrous meshes created by electrospinning from blends of PU with various concentrations of hydroxypropyl cellulose were also tested for transdermal drug delivery using donepezil hydrochloride, i.e., a drug used for treatment of Alzheimer disease [42]. PU was a component of a novel bilayer wound dressing, consisting of a commercial PU membrane as an outer layer, and an electrospun gelatin/keratin nanofibrous mat as an inner layer. The outer layer acted as a barrier against bacteria and other contaminants, while the inner layers promoted the adhesion, spreading, migration and growth of fibroblasts *in vitro*, and vascularization and wound healing in rats *in vivo* [23].

Polydimethylsiloxane (PDMS) in electrospun nanofibrous scaffolds was used in blends with thermoplastic PU (TPU) in 90:10, 80:20, and 70:30 blend ratios of TPU and PDMS. The activity of mitochondrial enzymes and proliferation of human dermal fibroblasts significantly increased with the percentage of PDMS in the scaffolds [14].

Polyethylene terephthalate (PET) in combination with honey improved the morphology of chitosan-containing fibers, decreased the diameter of electrospun fibers, increased the fiber deposition area in the collector, and increased the water uptake capacities of the material, which is important for exudating wounds [15]. In another study, low-molecular weight cationic compounds were synthesized from re-purposed PET and used for self-assembling into high aspect ratio supramolecular nanofibers for encapsulation and delivery of piperacillin/tazobactam (PT), an anionic antibiotic. In a *Pseudomonas aeruginosa*-infected mouse skin wound model, the treatment with the PT-loaded nanofibers was more effective in comparison with free PT, as evidenced by significantly lower counts of *P. aeruginosa* at the wound sites, and by a histological analysis [43].

Polyethersulfone (PES) nanofibrous membranes, made by fine tuning of electrospinning parameters, supported the proliferation of fibroblasts similarly as standard tissue culture polystyrene, and when applied as experimental wound dressings in mice, they showed a higher exudate absorption capacity, higher epithelial regeneration, greater fibroblast maturation, improved collagen deposition, and faster edema resolution than control commercial wound dressings, namely Vaseline gauze dressing and a conventional gas permeable bandage [16].

Polystyrene (PS) in its amorphous state is a transparent and colorless material. It is a hard, stiff, and very brittle polymer with remarkable water vapor permeability, very high electrical resistance, and low dielectric loss. For wound dressing applications, PS was electrospun with poly(ε-caprolactone) and chamomile extract, containing phenolics and flavonoids, particularly apigenin with remarkable wound healing effect [44]. Electrospun polystyrene nanofibrous scaffolds were also applied for cultivation of skin cells in dynamic bioreactors and at the air/liquid interface [45, 46].

Poly(acrylic acid) (PAA) was recently used for preparation of nanofibers incorporated with reduced graphene oxide, intended for delivery of antibiotics, which was controlled by photothermal activation of the nanofibers [18]. In another study, electrospun nanofibers consisting of PAA and poly(1,8-octanediol-*co*-citric acid), i.e., a synthetic biodegradable elastomer, showed intrinsic antibacterial activity and were used for topical delivery of physiologically relevant concentrations of growth factors [47].

Poly(methyl methacrylate) (PMMA) nanofibers were used for construction of antiscarring wound dressings. PMMA containing polyethylene glycol and kynurenic acid, an antifibrotic agent, suppressed proliferation of fibroblasts *in vitro*, and when administered as wound dressing in rats *in vivo*, they inhibited expression of collagen and fibronectin, and enhanced the production of matrix metalloprotease 1 (MMP-1), an ECM-degrading enzyme [48]. In addition, core-shell nanofibers containing PVA and PMMA were used for delivery of ciprofloxacin hydrochloride, an antibiotic [49].

Poly(di(ethylene glycol) methyl ether methacrylate) (PDEGMA), a thermoresponsive polymer, was blended with poly(L-lactic acid-*co*-ε-caprolactone), P(LLA-CL), and used for construction of nanofibrous carriers for controlled drug delivery, namely for delivery of ciprofloxacin. These fibers also supported the growth of fibroblasts, and by decreasing the temperature, they enabled the cell detachment and delivery into wounds [50].

Another thermoresponsive polymer, poly(N-isopropylacrylamide) (PNIPAM) was used for fabrication of nanofibers for transdermal delivery of drugs, namely levothyroxine (T_4), which helps to reduce deposits of adipose tissue [51].

3. Nanofibers from synthetic degradable polymers

Synthetic degradable polymers have been used as scaffolds for skin tissue engineering, but also as wound dressing releasing various bioactive molecules by a controllable manner. Degradable polymers typically used in these applications are aliphatic polyesters, such as poly-ε-caprolactone (PCL), polylactide (PLA), and poly(lactide-*co*-glycolide) (PLGA). These polymers were approved by the Food and Drug Administration of the United States of America (FDA) for many medical applications.

Poly-ε-caprolactone (PCL) has been used most frequently from the mentioned polymers. It is a semi-crystalline polymer with tunable mechanical properties, and has a good solubility in a variety of solvents, and hence it can be combined with variety of other polymers. In comparison to other polyesters, PCL is a slowly degrading polymer, which can be essential for specific applications [52], and products of its degradation are non-toxic in the nature [53]. The acidic products of polyester degradation can affect the healing processes after implantation and may lead to inflammation [54]. However, due to the slow degradation of PCL, this risk is significantly lower compared to PLA and PLGA, which degrade significantly faster [55]. Slower degradation of PCL in comparison with its copolymer with PLA (PLCL) was also confirmed in our study, where both polymers were exposed to enzymatic degradation using lipase and proteinase K enzymes [13] (**Figure 2**).

However, PCL is more hydrophobic than PLA and particularly PLGA, and thus it is less supportive for cell adhesion. Therefore, PCL was rarely electrospun alone, i.e., without other polymers and bioactive additives. Nevertheless, pure PCL nanofibrous scaffolds were successfully used for cultivation and differentiation of hair follicle stem cells, isolated from the bulge regions of rat whiskers [56, 57]. In addition, pure PCL scaffolds supported the proliferation of mesenchymal stem cells, fibroblasts, and keratinocytes better than pure PVA scaffolds [12]. In spite of this, for purposes of skin regenerative therapies, PCL was usually combined with natural polymers, such as collagen, which was either blended with PCL before electrospinning [6, 58], or deposited on PCL nanofibers [59]. Gelatin, a collagen-derived protein, was either blended with PCL [60], or incorporated into core-shell PCL/gelatin nanofibers as the core polymer [22]. Gelatin was also electrospun independently of PCL using a double-nozzle technique, which resulted in creation of two types of nanofibers in the scaffolds, either mixed [61] or arranged in separate gelatin and PCL layers [27]. Multilayered and blend structures were found to fit most of native skin requirements in comparison with all the other mentioned structures [27].

Other natural polymers for modification of PCL nanofibers included whey protein [62], hyaluronic acid [24], keratin [28, 63], chitosan [28], fibrinogen [64], or gum arabic, and a corn protein zein [65]. These natural polymers were blended with PCL in the electrospinning solution. Polymer-modified PCL nanofibrous scaffolds have been often further modified with growth factors, such as epidermal growth factor (EGF) immobilized on PCL/collagen nanofibers [58] or on PCL/gelatin nanofibers [60] and transforming growth factor-β1 (TGF-β1) added into PCL/collagen electrospinning solution [6].

Other bioactive substances used for incorporation into PCL-based nanofibers included medicinal herbs such as *Aloe vera* [61], lawsone, i.e., 2-hydroxy-1,4-naphthoquinone extracted from Henna, endowed with antimicrobial, antiparasitic,

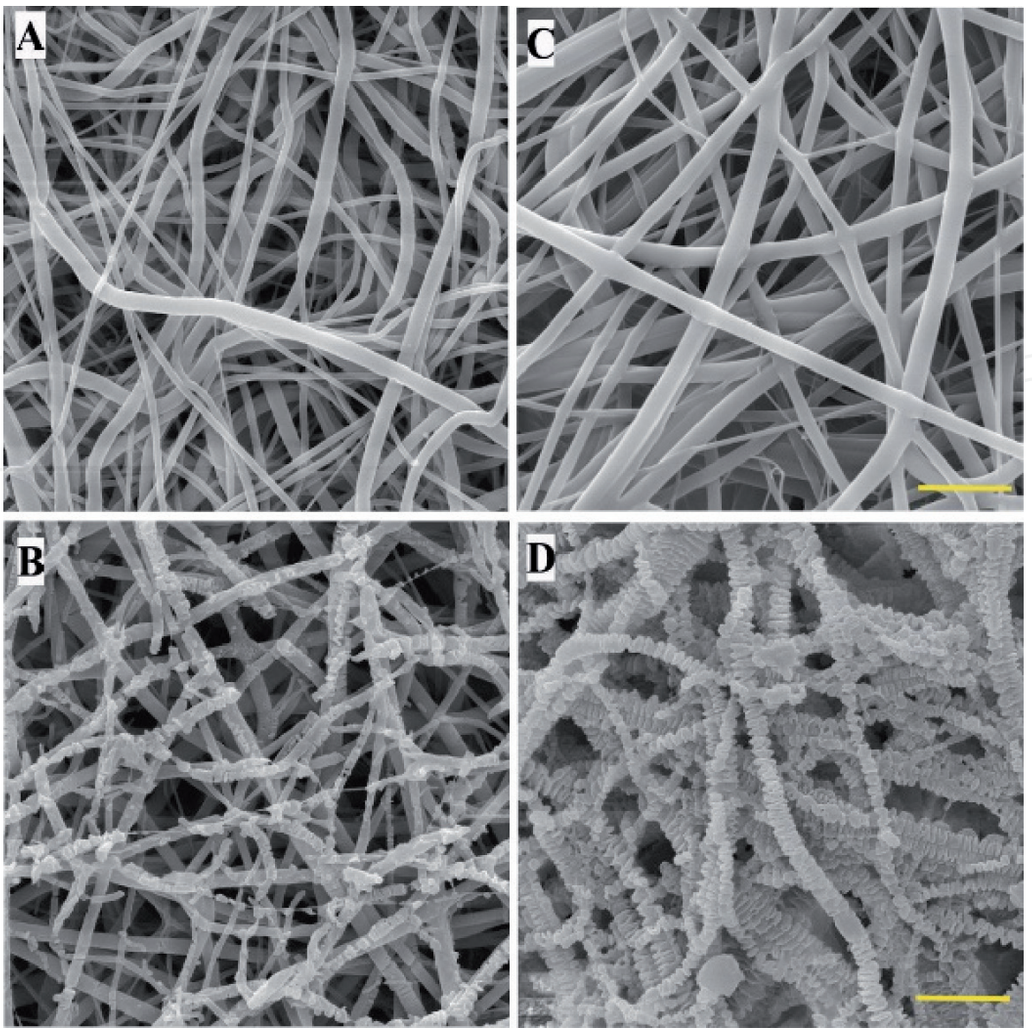

Figure 2.
Scanning electron microscopy analyses of electrospun PCL (A, B) and PLCL (C, D) in their intact state (upper row, A, C) and after 2 days of enzymatic degradation (lower row, B, D). Magnification 5000×, scale bar 10 μm.

anticancer, and antioxidant activities [22], other plant extracts with wound healing effects, e.g., from *Calendula officinalis* [65] or *Indigofera aspalathoides, Azadirachta indica, Memecylon edule*, *and Myristica andamanica* [66], molybdenum oxide nanoparticles for treating skin cancer [67] or antibiotics, which can be combined with PCL by various manners, e.g., through whey protein [62] or through micelles coating PCL/collagen nanofibers [6].

In our experiments, PCL electrospun nanofibrous membranes were impregnated with alaptide or L-arginine. Alaptide is a spirocyclic dipeptide, which was designed as an analogue of melanocyte-stimulating hormone release-inhibiting factor (MIF) and synthesized by Kasafirek et al. at the Research Institute for Pharmacy and Biochemistry in Prague, Czechoslovakia, in the 1980s of the twentieth century [68]. Alaptide showed a great potential for regeneration of the injured skin and also for enhanced transdermal penetration of drugs [69, 70]. Arginine is amino acid which is a precursor of nitric oxide, implicated in wound healing. Arginine promoted re-epithelization and vascularization of wounds [71] and supported proliferative, antiapoptotic, and immune defense functions of fibroblasts [72]. In our study, concentrations up to 2.5 wt.% of alaptide and up to 10 wt.% of L-arginine were used for fabrication of the membranes. Normal human dermal fibroblasts (NHDF) were cultivated on the membranes for 7 days. Alaptide-containing membranes were fully colonized by the cells up to the highest alaptide concentration (2.5 wt.%). However,

the highest arginine concentration (10 wt.%) appeared as cytotoxic (**Figure 3**). One of the possible explanations is a dual effect of nitric oxide, which can act either as antioxidant or as oxidative agent [73].

Polylactide (PLA) is a polymer obtained by the ring-opening polymerization of lactide, i.e., cyclic dimer of lactic acid, as the monomer. The lactide has two enantiomers, namely L-lactide and D-lactide. Polymerization of each enantiomer alone results in creation of poly-L-lactide (PLLA) or poly-D-lactide (PDLA). Polymerization of a racemic mixture of L-lactide and D-lactide, i.e., mixture containing equal amounts of both enantiomers, gives rise of poly DL-lactide (PDLLA).

For skin tissue engineering, similarly as in PCL, PLA was often combined with other polymers and biologically active molecules in order to tailor desirable properties of the scaffolds. For example, for enhancing the cell adhesion on nanofibrous PLA scaffolds, PLA was blended and electrospun together with gelatin. Composite scaffolds containing PLA and gelatin in a ratio of 7:3 were more suitable for the attachment and viability of fibroblasts than the scaffolds made either of PLA or of gelatin alone [9]. Similarly, composite nanofibrous scaffolds made by electrospinning of a blend of poly-L-lactic acid/poly-(α,β)-DL-aspartic acid/collagen (PLLA/PAA/Col I&III) increased the proliferation of adipose tissue-derived stem cells (ADSCs), i.e., an important cell type used in skin tissue engineering, in comparison with pure PLLA or PLLA/PAA scaffolds [74]. In our experiments, electrospun PLLA meshes were modified by additional coating with fibrin or collagen. Fibrin coating supported better the growth of dermal fibroblasts, while the growth of keratinocytes was better on collagen [10].

In another design of bilayer scaffolds for skin tissue engineering, PLLA in the form of microporous disc was combined with superficial chitosan/PCL nanofibrous mat. The disc was seeded with dermal fibroblasts, while the mat was used as

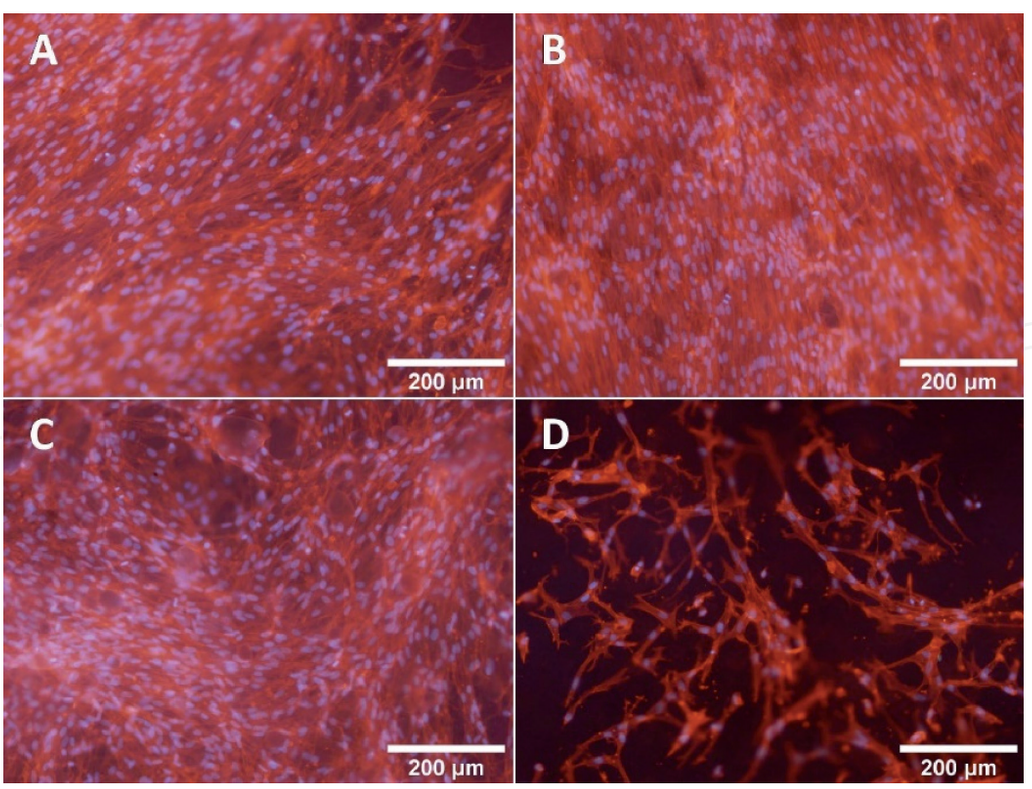

Figure 3.
Normal human dermal fibroblasts cultivated for 7 days on PCL nanofibrous membrane impregnated with alaptide or arginine. A—0.1 wt.% of alaptide, B—2.5 wt.% of alaptide, C—1 wt.% of arginine, 10 wt.% of arginine. The cells were stained for nuclei (blue) and actin (red) using DNA-binding dye DAPI and phalloidin conjugated with TRITC. The images were acquired using Olympus IX71 fluorescence microscope equipped with DP71 camera and lens 10× (N.A. = 0.3).

substrate for keratinocytes. The porous structure of the scaffolds allowed humoral communication of both cell types, but the nanofibers prevented the direct intermingling of these cell types [29].

Other interesting application of PLA nanofibers was skin tissue engineering for the infected wound site. PLA solution was electrospun together with highly porous silver microparticles (AgMPs) or high surface area silver nanoparticles (AgNPs) and used as substrates for co-culture of human epidermal keratinocytes and *Staphylococcus aureus*. The scaffolds with AgMPs showed a higher and steadier release of silver ions and lower cytotoxicity towards keratinocytes than AgNPs-loaded scaffolds [75].

PLA nanofibers have also been widely used for wound dressing applications, where they were loaded with various bioactive molecules improving wound healing and preventing microbial infection. Examples include PLLA/zein nanofibrous mats loaded with *Rana chensinensis* skin peptides with antibacterial and antioxidative activity [76], electrospun PLLA nanofibrous membranes coated by an *Aloe vera* gel [77], nanofibrillar matrices prepared from blends of PCL and PDLLA loaded with ciprofloxacin [78] or composite electrospun membranes containing polylactide:poly(vinyl pyrrolidone)/polylactide:poly(ethylene glycol) (PLA:PVP/PLA:PEG) core/shell fibers, designed for treatment of burns and loaded with curcumin and HHC36 antimicrobial peptides [8].

In spite of all these encouraging results, PLA and PCL can elicit inflammatory response. Although inflammation is the first physiological stage of wound healing, followed by proliferation and remodeling, excessive inflammation can delay the wound healing and can lead to ulceration, fibrosis, scar formation or entering the wound into a chronic state [79, 80]. The inflammatory response to PLA and PCL was reduced in electrospun co-axial scaffolds containing nanofibers with bioactive gelatin shells and biodegradable synthetic cores of PLA and PCL [81]. Another approach was the incorporation of PLA scaffolds with anti-inflammatory drugs. PLA nanofibers with 20 wt.% of ibuprofen promoted the viability and proliferation of human epidermal keratinocytes (HEK) and human dermal fibroblasts (HDF) *in vitro*, reduced wound contraction in mice *in vivo*, and when seeded with HEK and HDF, also enhanced new blood vessel formation in wounds of nude mice [80]. In a study by Yaru et al. [82], PLA nanofibers were incorporated with salicylate, a signaling molecule in plants, but also exhibiting a wide spectrum of signaling activities in mammals, including antithrombotic, anti-inflammatory, antineoplastic, and antimicrobial actions [83]. In addition, electrospun nanofibrous PDLLA scaffolds were incorporated with microalga *Spirulina*, which has anti-inflammatory, antioxidant, antimicrobial, antiallergenic, anticancer, and antidiabetic effects. The scaffolds were seeded with mesenchymal stem cells derived from mouse kidneys and used for treatment of the third degree burns in mice [79].

PLA and PCL can be combined in a poly(L-lactic acid-*co*-ε-caprolactone) copolymer, P(LLA-CL), also referred to as PLACL [84–86], PLLCL [26] or PLCL [13, 50].

As mentioned above, blend nanofibers of P(LLA-CL) and PDEGMA were prepared for controlled drug and cell delivery [50]. P(LLA-CL) was also blended with gelatin [84], silk fibroin, vitamin E, and curcumin [85] or with silk fibroin, tetracycline, and ascorbic acid [86], which increased the proliferation of human dermal fibroblasts on these nanofibrous scaffolds and secretion of collagen by these cells. Co-axial nanofibers with P(LLA-CL)/gelatin shell and albumin core containing EGF, insulin, hydrocortisone, and retinoic acid supported proliferation and epidermal differentiation of ADSCs better than nanofibers prepared by a blend spinning of all mentioned components [26]. In combination with poloxamer (Pluronic) 123,

P(LLA-CL) was also used for electrospinning of nanofibrous scaffolds for direct delivery of ADSCs into wounds in order to promote their healing [87].

In our experiments, composite PCL/PLCL nanofibers were coated either with platelet lysate, or with platelet lysate incorporated in fibrin assemblies [88] in various concentrations. Results for human keratinocytes (HaCaT cells) indicated that the presence of platelet lysate increased the metabolic activity and phenotypic maturation of keratinocytes. The best results were observed when the nanofibers were coated with fibrin together with platelet lysate (**Figure 4**).

Poly(lactide-*co*-glycolide) (PLGA) is a copolymer obtained by the ring-opening co-polymerization of two different monomers, i.e., lactic acid and glycolic acid. In skin regenerative therapies, it was applied for both skin tissue engineering and wound dressing. For these applications, PLGA was combined with various natural and synthetic polymers and bioactive compounds. For example, using bovine serum albumin as a carrier protein, vitamin C, vitamin D3, hydrocortisone, insulin, triiodothyronine, and EGF were simultaneously blend-spun into PLGA-collagen nanofibers. All these factors concertedly increased proliferation of fibroblasts and keratinocytes, while maintaining the keratinocyte basal state. In addition, vitamin C maintained its ability to facilitate secretion of type I collagen by fibroblasts, EGF stimulated proliferation of skin fibroblasts, and insulin potentiated adipogenic differentiation of fibroblasts [11]. In PLGA nanofibers, EGF was also combined with the local anesthetic lidocaine in order to accelerate wound healing in a rat model [89]. Coating PLGA nanofibers with a self-assembled complex of poly(ethylene argininyl aspartate diglyceride) polycation, heparin, and cargo growth factors, i.e., vascular endothelial growth factor (VEGF) and/or transforming growth factor-beta3 (TGF-β3), enhanced proliferation of human dermal fibroblasts and formation of tubular structures from human umbilical vein endothelial cells *in vitro*. In addition, these nanofibers reduced necrosis, improved vascularization, and maintained well-composed skin appendages in a mouse skin flap model *in vivo* [25]. Growth factors, namely recombinant human EGF and recombinant human basic fibroblast growth factor (bFGF), were also encapsulated in PLGA microspheres and loaded into hybrid scaffolds of PLGA and polyethylene oxide [90]. Both growth factors had a synergistic effect on the proliferation of human skin fibroblasts and increased the expression of genes for collagen and elastin in these cells [90]. Composite nanofibrous membranes containing PLGA and cellulose nanocrystals and loaded with neurotensin accelerated healing of full-thickness skin wounds in spontaneously diabetic mice [91]. Nanofibers created by electrospinning the dispersion composed of polyethyleneimine-carboxymethyl chitosan/pDNA-angiogenin

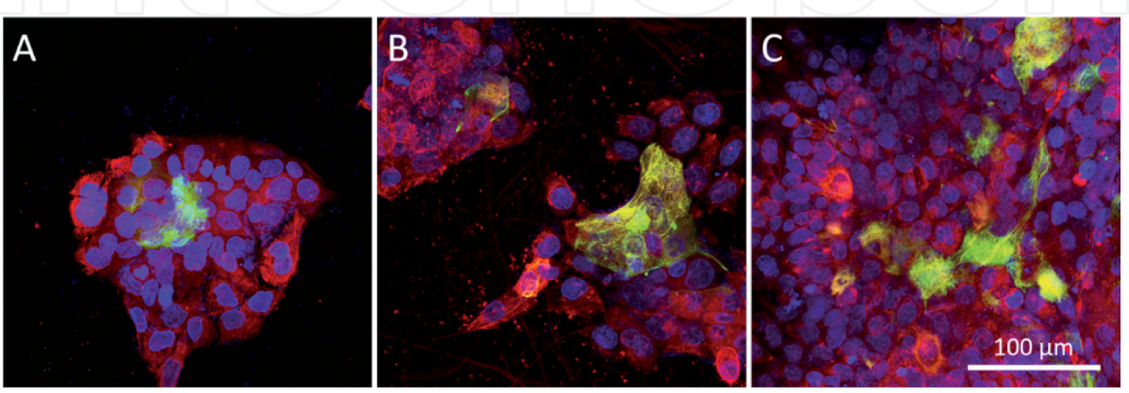

Figure 4.
Immunofluorescence staining of cytokeratin 10 (green), cytokeratin 14 (red), and nuclei (blue) of HaCaT cells after 7 days in culture grown on PCL/PLCL nanofibers. Cell on nanofibers without coating (A), coated with platelet lysate (B) and coated with fibrin assemblies with platelet lysate (C) are shown. Leica TCS SPE DM2500 confocal microscope.

nanoparticles, curcumin, PLGA, and cellulose nanocrystals showed antimicrobial and regenerative effects when transplanted into the infected full-thickness burn wounds in rats [92].

The PLGA nanofibers were also modified with ECM components. In a study by Shtrichman et al. [93], the PLGA nanofibrous scaffolds were modified with ECM deposited on these scaffolds by mesenchymal progenitor cells, derived from human embryonic stem cells, and human induced pluripotent stem cells, originating from hair follicle keratinocytes, which were cultured on the scaffolds and removed by subsequent decellularization. Subcutaneous implantation of the ECM-modified scaffolds in rats then showed that this stem cell-derived construct is biocompatible, biodegradable, and holds great potential for tissue regeneration applications. In addition, ECM-derived proteins, such as collagen and gelatin, can be electrospun directly together with PLGA [94]. In our earlier study, PLGA nanofibers were modified with fibrin or collagen in a similar manner as PLLA [10]. The morphology of these coatings, and also the behavior of HaCaT keratinocytes and human dermal fibroblasts on the coated and uncoated nanofibers, were similar on PLGA and PLLA [10].

PLGA-based nanofibrous meshes were also used for treatment of skin fibrosis and keloids, formed by abnormal proliferation of scar tissue at the site of cutaneous injury. Composite nanofibers of PLGA and poly(vinyl alcohol) loaded with kynurenine, a tryptophan metabolite, improved the dermal fibrosis in a rat model [95]. PLGA nanofibers releasing dexamethasone and green tea polyphenols significantly induced the degradation of collagen fibers in keloids on the back of nude mice [96].

Last but not least, PLGA nanofibers were also explored for transdermal delivery of drugs with poor oral absorption and limited bioavailability, e.g., Daidzein, a promising candidate for treating cardiovascular and cerebrovascular diseases [97], or for local delivery of anticancer drugs (for a review, see [98]).

Another important degradable polymer for fabrication of nanofibrous meshes for skin regenerative therapies is poly(ethylene glycol) (PEG), also known as poly(ethylene oxide) (PEO), depending on its molecular weight. PEG usually refers to polymers with a molecular mass below 20,000 g/mol, while PEO refers to polymers with a molecular mass above 20,000 g/mol.

In nanofibrous scaffolds, PEG or PEO have been usually used as auxiliary components improving electrospinnability, mechanical properties, and wettability of other polymers. For example, PEO was used to enable electrospinning of casein (i.e., a protein extensively used for drug delivery), which does not possess sufficient viscoelasticity due to its extensive intermolecular interactions [99], or to improve the electrospinnability and mechanical properties of silk fibroin [100]. As mentioned above, PEO or PEG was electrospun together with PMMA for creation of nanofibers delivering kynurenic acid [95] or with PLGA for delivery of human recombinant EGF and bFGF [90]. Other interesting applications of PEO include creation of electrospun carboxymethylcellulose/PEO nanofibers for delivery of viable commensal bacteria for preventive diabetic foot treatment [101], creation of three-dimensional scaffolds composed of PCL-PEG-PCL tri-block copolymer and iron oxide (Fe_3O_4) nanoparticles for skin tissue engineering [102], creation of biodegradable nanofiber mats based on thermoresponsive multiblock poly(ester urethane)s comprising PEG, poly(propylene glycol) (PPG), and PCL, which showed improved hydrolytic degradation compared to pure PCL and excellent adhesion of human dermal fibroblasts [103]. The adhesion and growth of fibroblast were also improved after combination of PLCL with Pluronic, i.e., a copolymer of PEO and poly(propylene oxide) (PPO) arranged in a tri-block PEO-PPO-PEO structure [104].

Other auxiliary polymers used for creation of nanofibrous scaffolds are poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP). In some studies,

PVA is regarded as hydrolytically degradable [17, 105], while in other studies, it is considered non-degradable [106]. PVP has been reported to be hydrolytically degradable [105]. In addition, both PVA and PVP are hydrophilic and water soluble, and thus they can be removed from a composite polymeric mesh in water environment. This property of PVA, PVP, and also of PEG or PEO, can be used for creation of so-called "sacrificial fibers" in order to enlarge the pores in nanofibrous scaffolds for penetration of cells [107]; for a review, see [108, 109] or for tailoring the appropriate surface roughness of nanofibers inside the scaffolds. For example, PLLA was electrospun together with PVP in increasing concentrations, and after subsequent etching of PVP from the scaffolds in water environment, nano- and microfibers with increasing nanoscale surface roughness were obtained. Higher surface nanoroughness and porosity of PLLA fibers increased their hydrophilicity and their colonization with human dermal fibroblasts [32]. Other applications of PVA and PVP are similar to those of PEG or PEO, i.e., to increase spinnability of poorly spinnable substances used for skin regenerative therapies. For this purpose, PVA was combined with polysaccharides, such as gum tragacanth [110] or Schizophyllan [111], and PVP with *Aloe vera* [112]. Both PVA and PVP have been used to improve mechanical properties, wettability, and attractiveness for cell adhesion of various synthetic and natural polymers, particularly PCL [110] and chitosan [1]. PVA was used as emulsifier in fabrication of blended electrospun PLGA/chitosan nanofibers for potential skin reconstruction [113]. PVA and particularly PVP are important components of nanofibers delivering various biomolecules and drugs into skin, such as antibiotics (PVA [49], PVP [114]), kynurenine (PVA [48]), curcumin and HHC36 antimicrobial peptides (PVP [8]) or antimicrobial suberin fatty acids isolated from outer birch bark (PVP [115]). PVA and PVP were combined in electrospun nanofibrous membranes designed for sustained release of the antibiotic ciprofloxacin into wounds [116]. Nanofibers with cellulose acetate (CA) as the core material and PVP solution as the shell material were used for transdermal delivery of artemisinin, a potent antimalarial drug, which was incorporated into CA [117].

4. Advanced skin tissue engineering

4.1 Dynamic bioreactors

Tissue engineering in general, including skin tissue engineering, can be markedly improved by cultivation of cell-material constructs in dynamic bioreactors. These systems not only improve the supply of oxygen and nutrients to cells and waste removal, but also mechanically stimulate the cells with positive effects on their growth, differentiation, and phenotypic maturation.

First of all, the cell seeding can be improved in dynamic systems. In a study by Vitacolonna et al. [118], various methods of seeding fibroblasts on acellular dermal matrix were compared, namely static cell seeding after previous degassing of the matrix using a low-pressure syringe system, orbital shaker seeding, centrifugal seeding, and their combinations. Centrifugal seeding combined with matrix degassing significantly increased the seeding efficiency and homogeneity compared to the other methods.

Also the subsequent proliferation and other performance of cells can be markedly influenced by the dynamic cultivation. For example, human epidermal stem cells cultured on microcarriers in a rotary bioreactor exhibited higher proliferation and viability than the cells cultured in static conditions [119]. Human fibroblasts on nanofibrous poly(3-hydroxybutyrate-co-3- hydroxyvalerate) (PHBV) scaffolds, subjected to biaxial distension for periods of time in a dynamic bioreactor,

developed elastin fibers, whereas the cells on the same scaffolds cultured under static conditions showed negligible elastin production [120]. Cyclic uniaxial stretching of human HaCaT keratinocytes on collagen-silicon sheets induced the production of metalloproteinase 9 (MMP-9), a proteolytic enzyme necessary for keratinocyte migration, in these cells [121]. Strain also improved the mechanical strength of an engineered skin containing electrospun collagen scaffolds, human dermal fibroblasts, and epidermal keratinocytes, which was probably a result of enhanced epidermal cell proliferation, differentiation, and increased ECM production [34]. The keratinocyte differentiation under mechanical tension can be attributed to up-regulation of h2-calponin, which associates with actin stress fibers and decreases the cell proliferation rate (for a review, see [122]). Another type of mechanical stimulation implicated in keratinocyte differentiation is pressure stress, which increases the concentration of intracellular calcium, a stimulator of keratinocyte differentiation [35, 123].

In our experiments, we have developed a custom perfusion dynamic culture system allowing cell cultivation on elastic silicone membranes and generating cyclic pressure stress. First, these membranes were treated with plasma in order to increase their wettability and their ability to attach thin films made of nanofibrillar cellulose [124]. Afterwards, the porcine adipose tissue-derived stem cells were seeded on this surface. After 7 days of mechanical stimulation, a multilayered cell structure was observed in dynamic conditions, whereas in static conditions, only a cell monolayer was formed (**Figure 5**).

Increased concentration of calcium in keratinocytes and their differentiation can be also achieved by other means than mechanical stimulation, namely by stimulation with laser beam [125] or monodirectional pulsed electric current [126]. Electrical stimulation also enhanced the migration and proliferation of fibroblasts,

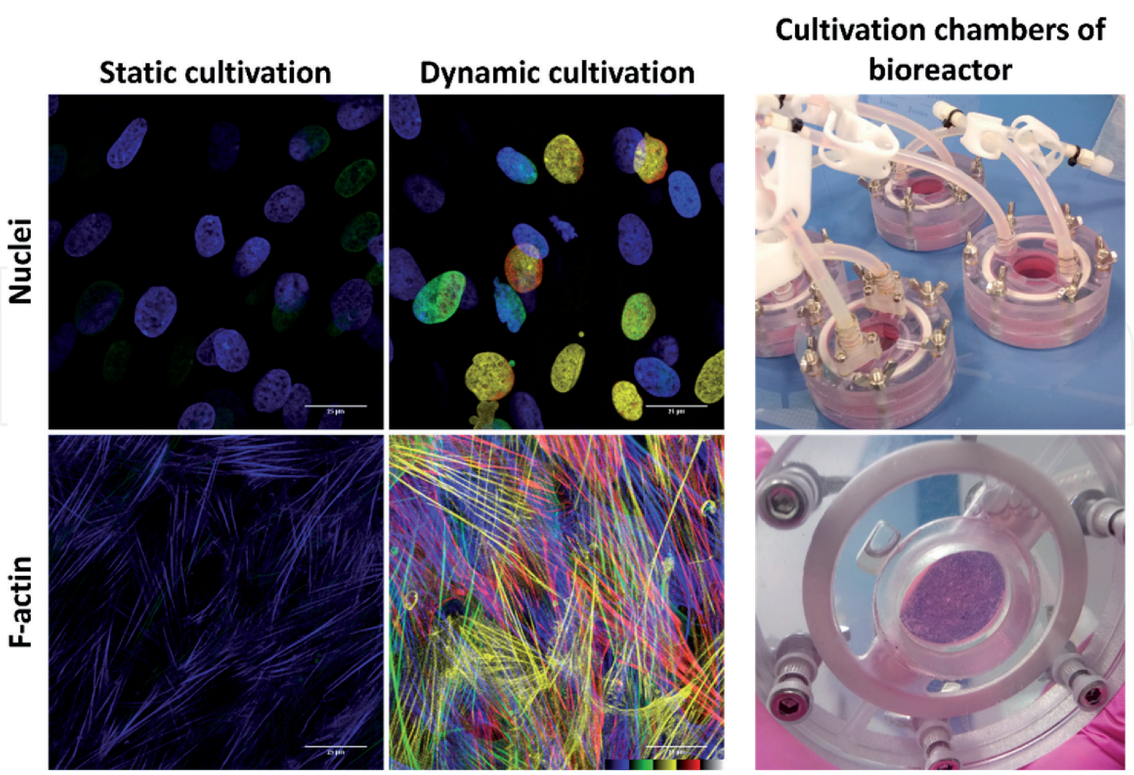

Figure 5.
Color-coded projection of porcine adipose tissue-derived stem cells cultivated on thin nanocellulose film structure in static (left) and dynamic conditions (middle). Fluorescence staining of nuclei (DAPI) and F-actin (Phalloidin). Right: Custom built culture chambers creating controlled mechanical stress and strain with perfusion. Below, formation of a multilayered structure of cells creating opaque layer on the nanocellulose-coated silicone membrane.

expression of ECM proteins in these cells, and differentiation of these cells towards myofibroblasts, i.e., processes critical for wound healing [127]. The positive effect of electrical current on fibroblasts can be further combined with light stimulation of the fibroblast proliferation, e.g., on nanofibrous PCL scaffolds electrospun with a semiconductive polymer, namely poly(N,N-bis(2-octyldodecyl)-3,6-di(thiophen-2-yl)-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione-alt-thieno[3,2-b]thiophene) (PDBTT), subjected to the illumination from a red light-emitting diode [128]. Also magnetic stimulation can be effectively used in skin tissue engineering. For example, multilayered sheets of keratinocytes were obtained by cultivation of keratinocytes loaded with magnetite cationic liposomes in a magnetic field. After removal of the magnet, the sheets were released from the cultivation plates, and were harvested with a magnet. This technology was termed "magnetic force-based tissue engineering" [129].

4.2 Air-liquid interface

In most experimental studies dealing with skin tissue engineering *in vitro*, keratinocytes are submerged in the cell culture media. However, in physiological skin *in vivo*, keratinocytes are exposed to air, at least their uppermost layer, i.e., *stratum corneum*, which is an impermeable barrier of cornified cell layers. Therefore, in advanced tissue engineering, keratinocytes should be exposed to the air-liquid interface (**Figure 6**) in order to achieve their phenotypic maturation and creation of the *stratum corneum* and the other epidermal layers, namely the basal, spinous, and granular layers [130].

The early differentiation of human amnion epithelial cells towards keratinocytes, manifested by formation desmosomes, was more pronounced in cells cultured at the air-liquid interface than in cells submerged in the culture medium [131]. In another study, human ADSCs were transdifferentiated towards keratinocytes in a medium containing retinoic acid, hydrocortisone, ascorbic acid, and bone morphogenetic protein-4 (BMP-4). This medium enabled high expression of pan-cytokeratin in conventional 2D cultures, especially if the cells were grown on type IV collagen. When the cell cultures were lifted to air-liquid interface, significant stratification was observed, particularly on growth supports coated with type IV collagen or fibronectin, and epidermal differentiation markers, such as involucrin and cytokeratins 1 and 14, were induced [132].

At the air-liquid interface, the keratinocytes or cells differentiating towards keratinocytes have been cultured on various substrates, e.g., acellular dermis [133], porous sponge-like gelatin scaffolds incorporated with chrondroitin-6-sulfate and hyaluronic acid [134], de-epithelialized human amniotic membrane [135], collagen IV and fibronectin [132] and fibrin in the form of layer [131], hydrogel or clot [136]. On the mentioned substrates, keratinocytes were grown either alone or in combination with fibroblasts submerged in the culture medium. In a study by Wang et al. [134], fibroblasts were grown inside the porous gelatin-based scaffolds submerged in the medium, while keratinocytes were grown on the top of the scaffolds, exposed to the air-liquid interface. Similarly, on the de-epithelialized amniotic membrane,

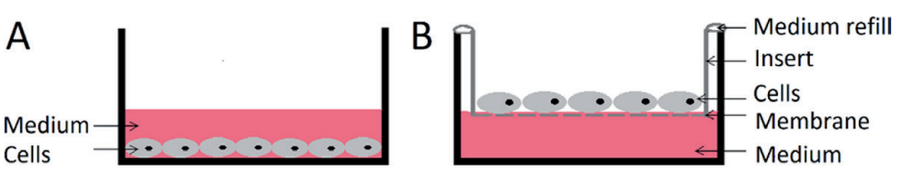

Figure 6.
The principle of cell cultivation in a conventional cell culture system (A) and at the air-liquid interface (B).

fibroblasts were cultured on the lower side of the membrane, submerged in the culture medium, while keratinocytes were grown on the upper side at the air-liquid interface [135]. In a study by Keck et al. [136], even a three-layered skin substitute was created. For the hypodermal layer, ADSCs and mature adipocytes were seeded within a fibrin hydrogel. On this layer, a fibrin clot with incorporated fibroblasts was placed for construction of the dermal layer. Keratinocytes were then added on the top of the two-layered construct and cultured at the air-liquid interface in order to create the epidermal layer [136].

Regarding the use of nanofibrous scaffolds for cultivation of keratinocytes at the air-liquid interface, synthetic and nature-derived scaffolds were used, namely electrospun PCL scaffolds [137, 138], electrospun polystyrene scaffolds [45] and fibrous sheets obtained after culturing human fibroblasts with ascorbic acid [139].

PCL scaffolds were used for construction of a three-dimensional *in vitro* skin model. The scaffolds were seeded with keratinocytes and melanocytes isolated from human scalp skin and cultured at the air-liquid interface. The keratinocytes contained a number of keratin fibrils and membrane-coated granules and formed a multilayered concentric structure, the surface of which became distinctly keratinized at the air-liquid interface. Cells with characteristic of melanocytes showed scattered distribution within the construct [137]. PCL scaffolds loaded with wound healing drugs, namely dexpanthenol and metyrapone, were used for a cell-based wound healing assay for rapid and predictive evaluation of wound therapeutics *in vitro*, using human HaCaT keratinocytes cultured at the air-liquid interface [138].

Interesting results were obtained on electrospun polystyrene scaffolds. In the absence of serum, keratinocytes, fibroblasts, and endothelial cells did not grow when cultured alone. However, when fibroblasts were cocultured with keratinocytes and endothelial cells, expansion of keratinocytes and endothelial cells occurred even in the absence of serum. Furthermore, the cells displayed native spatial three-dimensional organization when cultured at the air-liquid interface, even when all three cell types were introduced at random to the scaffolds [45].

The fibrous sheets produced by fibroblasts were used for creation of reconstructed human skin *in vitro*. After seeding the sheets with keratinocytes and the cell maturation *in vitro*, the reconstructed skin exhibited a well-developed human epidermis that expressed differentiation markers and basement membrane proteins [139].

The cultivation of keratinocytes at the air-liquid interface was also combined with cultivation of these cells in dynamic bioreactors, which further improved their growth and phenotypic maturation. Uniaxial strain stress (deformation of the cultivation substrate by 5–20%) further enhanced proliferation and epidermal differentiation of keratinocytes cultured at the air-liquid interface on electrospun collagen scaffolds containing fibroblasts in comparison with keratinocytes on unstrained cell-material constructs [34].

Also the perfusion with cell culture media showed beneficial effects on tissue-engineered skin constructs at the air-liquid interface. In a perfusion system with various growth supports for cells, such as acellular human dermis, Azowipes, electrospun polystyrene, and an electrospun composite of polystyrene and poly-DL-lactide fibers, human dermal fibroblast and endothelial cells showed greater viability under submerged conditions than at the air-liquid interface, whereas keratinocytes favored cultivation at the air-liquid interface. In addition, the viability of keratinocytes and fibroblasts was higher under continuous perfusion than under batch-feed perfusion, and on electrospun scaffolds than on acellular dermis [46]. In a recent study, a reconstructed skin model *in vitro*, containing a collagen matrix incorporated with fibroblasts and keratinocytes cultured at the air-liquid interface, was exposed to a continuous flow of cultivation medium (from 1.25 to

7.5 ml/h) at its basal side. Histological examination confirmed the formation of a significantly thicker *stratum corneum* compared to the control constructs cultivated under static conditions. Moreover, the keratinocyte differentiation markers involucrin and filaggrin, as well as the tight junction proteins claudin 1 and occludin, showed increased expression in the dynamically cultured skin models. However, the skin barrier function of the dynamically cultivated skin models was not enhanced compared with the skin models cultivated under static conditions [36]. Similar results were obtained in a study by Kalyanaraman et al. [140], performed on engineered skin substitutes based on collagen-glycosaminoglycan sponges, containing fibroblasts in their inside and keratinocytes on their surface, which were exposed to the air-liquid interface. Perfusion of these construct with the medium at the flow rate of 5 ml/min increased the metabolic activity of fibroblasts and maintained the epidermal barrier created by keratinocytes similarly as in static controls, while higher flow rates of 15 ml/min, and particularly 50 ml/min, decreased the cell metabolic activity, increased the degradation of the scaffolds and decreased the epidermal barrier function, manifested by its increased hydration [140].

5. Conclusions

Nanofibrous scaffolds made of synthetic polymers have been widely investigated for their potential use in skin regenerative therapies. Non-degradable polymers used for preparation of nanofibrous scaffolds included polyurethane (which can also be prepared in degradable form), polydimethylsiloxane (PDMS), polyethylene terephthalate (PET), polyethersulfone (PES), and even polystyrene (PS). These scaffolds were mainly intended for wound dressing applications, and in case of PS, also for cultivation of skin cells in dynamic bioreactor and at the air/liquid interface. For creation of nanofibrous meshes, the non-degradable polymers have been often used in combinations with nature-derived polymers (dextran, chitosan, gelatin, and keratin), and loaded with various wound healing, angiogenic, antioxidant, anti-inflammatory, photoprotective, and antimicrobial substances. Non-degradable synthetic polymers also include hydrogels, such as poly(acrylic acid) (PAA), poly(methyl methacrylate) (PMMA) and particularly poly(di(ethylene glycol) methyl ether methacrylate) (PDEGMA), which is thermoresponsive and suitable for controlled drug delivery and cell delivery into wounds. Degradable synthetic polymers have been also applied in wound healing, but also as direct scaffolds for skin tissue engineering, i.e., as carriers for keratinocytes, fibroblasts, and stem cells. The most widely used degradable polymers for these applications include polycaprolactone (PCL) and its copolymers with polylactides (PLCL), and also polylactides (PLLA and PDLLA) and their copolymers with polyglycolides (PLGA). Similarly as non-degradable polymers, also degradable polymers are almost exclusively used in combination with nature-derived polymers (collagen, gelatin, keratin, fibrin, and glycosaminoglycans) in order to increase their attractiveness for cell colonization, and also with some synthetic polymers, such as poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO), poly (vinyl alcohol) (PVA), and poly(vinyl pyrrolidone) (PVP). These synthetic polymers act as auxiliary, i.e., improving electrospinnability, mechanical properties, and wettability of other polymers. Similarly as non-degradable polymers, also degradable polymers have been loaded with a wide range of growth and angiogenic factors and other biologically active substances. The cell performance on non-degradable and degradable nanofibrous scaffolds can be further markedly improved by cultivation in dynamic bioreactors and/or at air/liquid interface.

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