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Chapter

Nanofibrous Scaffolds for Skin Tissue Engineering and Wound Healing Based on Nature-Derived Polymers

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Abstract

Nanofibrous scaffolds belong to the most suitable materials for tissue engineering, because they mimic the fibrous component of the natural extracellular matrix. This chapter is focused on the application of nanofibers in skin tissue engineering and wound healing, because the skin is the largest and vitally important organ in the human body. Nanofibrous meshes can serve as substrates for adhesion, growth and differentiation of skin and stem cells, and also as an antimicrobial and moisture-retaining barrier. These meshes have been prepared from a wide range of synthetic and nature-derived polymers. This chapter is focused on the use of nature-derived polymers. These polymers have good or limited degradability in the human tissues, which depends on their origin and on the presence of appropriate enzymes in the human tissues. Non-degradable and less-degradable polymers are usually produced in bacteria, fungi, algae, plants or insects, and include, for example, cellulose, dextran, pullulan, alginate, pectin and silk fibroin. Well-degradable polymers are usually components of the extracellular matrix in the human body or at least in other vertebrates, and include collagen, elastin, keratin and hyaluronic acid, although some polymers produced by non-vertebrate organisms, such as chitosan or poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), are also degradable in the human body.

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

1. Introduction

Nanofibrous scaffolds are one of the most promising materials for skin tissue engineering and wound dressing, because they resemble nanoarchitecture of the native extracellular matrix (for a review, see [1]). Therefore, they can serve as suitable carriers of cells for tissue engineering and also as suitable wound dressings, which are able to protect the wound from external harmful effects, mainly

microbial infection, and at the same time, they can keep appropriate moisture and gas exchange at the wound site.

Nanofibrous scaffolds for skin tissue engineering have been fabricated from a wide range of synthetic and nature-derived polymers, which can be either biostable or degradable within the human body. Biostable synthetic polymers used in nanofiber-based skin regenerative therapies include, for example, polyurethane [2], polydimethylsiloxane [3], polyethylene terephthalate [4], polyethersulfone [5], and also hydrogels such as poly(acrylic acid) (PAA, [6]), poly(methyl methacrylate) (PMMA, [7]), and poly[di(ethylene glycol) methyl ether methacrylate] (PDEGMA, [8]). Degradable synthetic polymers typically include poly(ε-caprolactone) (PCL, [9]) and its copolymers with polylactides (PLCL, [10]), polylactides (PLA, [11]) and their copolymers with polyglycolides (PLGA, [12]), and also so-called auxiliary polymers, such as poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO, [13]) or poly(vinyl alcohol) (PVA, [14]), which facilitated the electrospinning process and improved the mechanical properties and wettability of the chief polymer. However, the synthetic polymers, although they are well-chemically defined and tailorable, are often bioinert, hydrophobic and thus not promoting cell adhesion, and also not well-adhering to the wound site. Therefore, they need to be combined with other bioactive substances, particularly nature-derived polymers.

This chapter is focused on nature-derived polymers used for fabrication of nanofibrous scaffolds for skin tissue engineering and wound healing. The advantages of most of these polymers are their better bioactivity, flexibility, wettability, and adhesion to the wound site. Similarly as synthetic polymers, also nature-derived polymers can be divided into polymers with none or limited degradability, when implanted into human tissues, and polymers well-degradable in human tissues. The first group includes glucans, such as cellulose, schizophyllan, dextran, starch, and other polysaccharides and proteins, such as pullulan, xylan, alginate, pectin, gum tragacanth, gum arabic, silk fibroin, and sericin. The second group of polymers degradable in human tissues includes collagen and its derivative gelatin, elastin, keratin, glycosaminoglycans such as hyaluronic acid, heparin and chondroitin sulfate, and also polymers not produced in the human body, namely chitosan, gellan gum, zein, and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV).

Some of the polymers degradable in human tissues, such as collagen, gelatin, elastin, keratin, and glycosaminoglycans, contain specific cell-binding motifs in their molecules, for example, specific amino acid sequences in proteins and oligosaccharide domains in glycosaminoglycans, which are recognized by cell adhesion receptors of integrin and non-integrin families (for a review, see [15, 16]). These molecules are often used in allogeneic or xenogeneic form, thus they can be associated with pathogen transmission or immune reaction. However, some synthetic polymers, for example PLA and PCL, have been reported to induce a more pronounced inflammatory reaction than gelatin [17].

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

2. Nature-derived nanofibers with none or limited degradability in the human tissues

Nature-derived nondegradable polymers or polymers with limited degradability in human tissues include polymers not occurring in the human body and synthesized by other organisms, such as plants, algae, fungi, insects, and bacteria.

Cellulose is a typical natural polymer nondegradable in human tissues. Cellulose belongs to the group of glucans, that is, polysaccharides derived from D-glucose, linked by glycosidic bond. In the cellulose molecules, these glycosidic bonds are of the β-type, thus the cellulose is a β-glucan. It is structural polysaccharide consisting of a linear chain of several hundred to over ten thousand β(1 → 4) linked D-glucose units. Cellulose is synthesized by plants, algae, fungi, some species of bacteria (*Gluconacetobacter xylinus*), and also by some animals, namely tunicates (*Styela clava*) (for a review, see [18, 19]).

Nanofibrous cellulose can be prepared in three basic forms: bacterial cellulose, which contains cellulose nanofibrils, synthesized by bacteria, nanofibrillar cellulose prepared from plants, particularly from wood, by hydrolysis, oxidation, and mechanical disintegration, and cellulose nanofibers created by electrospinning (for a review, see [19]). For electrospinning, cellulose should be solved. Well-known solvent of cellulose is N-methylmorpholine-N-oxide (NMMO). Another possibility is N-alkylinidazolium-derivate ionic liquid and N,N-dimethylacetamide containing 8 wt% of LiCl. However, any of them did not prove to be a good solvent for needleless electrospinning. The most favorable solvent of cellulose was found to be trifluoroacetic acid (TFA). However, TFA causes severe skin burns and is toxic for aquatic organisms even in low concentrations [20]. These problems, which limit the use of cellulose for creation of electrospun scaffolds for biomedical applications, can be solved by substituting the natural cellulose by its derivatives. The mostly used derivative of cellulose is cellulose acetate (CA), mainly due to its easier solubility and biocompatibility. CA can be dissolved in several solvents, however the best ones for electrospinning proved to be acetic acid (AA), and mixtures of acetone and N,N-dimethylacetamide (DMAC). Some results of successfully spun fibers by needleless electrospinning in our experiments can be found in **Figure 1**, demonstrating differences in the fiber morphology. The 95% aqueous mixture of AA showed the best results in comparison with acetone/DMAC mixtures due to production of smoother fibers and lower cytotoxicity.

All the mentioned forms of cellulose have been widely applied as wound dressings releasing various bioactive agents into wounds (antimicrobial, anti-inflammatory, antioxidative agents, cytokines, and growth and angiogenic factors), as transparent wound dressings for direct optical monitoring of wounds, for systemic

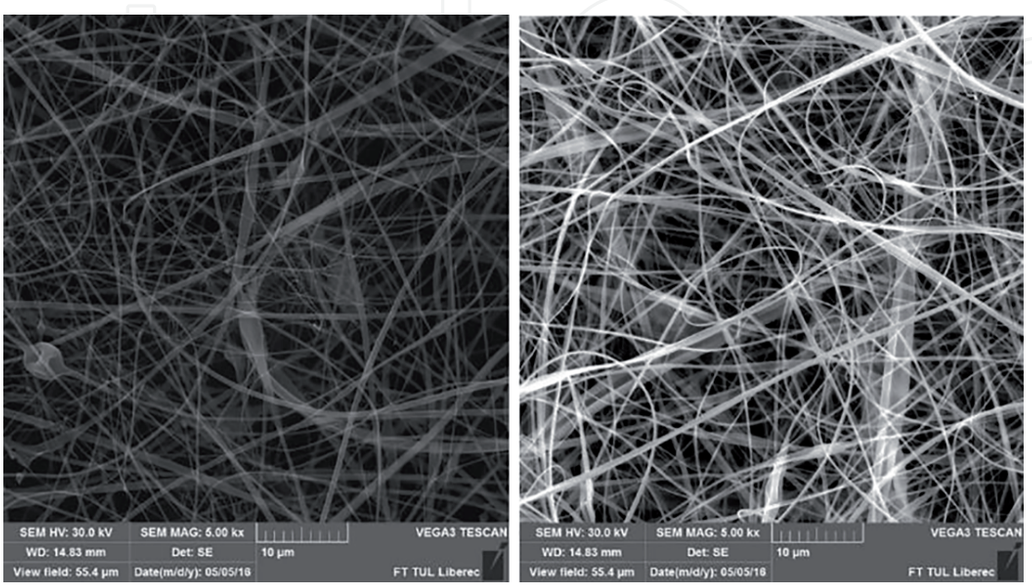

Figure 1.
Scanning electron microscopy of nanofibrous layers produced by wire needleless electrospinning using different solvents, namely 12 wt% of CA in acetone/DMAC (9:1) (left) or 14 wt% of CA in 95% AA (right).

transdermal drug delivery (analgesics, antiphlogistics, corticoids, and antihypertensives) and for construction of epidermal electronics for monitoring wound healing or physiological status of the organism. Non-degradable nanocellulose has also been used as a temporary carrier for delivery of keratinocytes, dermal fibroblasts, and mesenchymal stem cells into wounds (for a review, see [19]).

However, for use as direct scaffolds for skin tissue engineering, cellulose should be rendered degradable in human tissues. Cellulose is degradable by cellulase enzymes (exoglucanases and endoglucanases), which hydrolyze 1,4-beta-D-glycosidic linkages. These enzymes are not synthesized in human tissues, but they can be incorporated into cellulose scaffolds in order to degrade them gradually [21, 22]. These enzymes are believed to be non-toxic for mammalian cells [23, 24]. Moreover, the final product of cellulose degradation by these enzymes is glucose, which is a natural nutrient for the cells, by contrast with the acidic by-products of the standard currently used biodegradable PLA or PLGA scaffolds [25]. Another possibility how to use cellulase enzymes in skin tissue engineering (and in tissue engineering in general) is cell sheet technology. First, cells can be grown on the top of non-degradable cellulose substrates. After reaching the cell confluence, self-standing cell sheets can be released by exposure of the cellulose substrates to cellulases. Unlike the proteolytic enzymes conventionally used for detaching cells from their growth supports, cellulases do not disintegrate the extracellular matrix (ECM) formed by cells and do not cleave extracellular parts of cell adhesion receptors binding the ECM [26]. The cell sheets can be then replanted in the wound bed.

Another interesting approach how to render the cellulose degradable was metabolic engineering of *Gluconacetobacter xylinus*, which then produced modified cellulose molecules with intercalated N-acetylglucosamine (GlcNAc) residues, susceptible to degradation with lysozyme, present in the human body. After subcutaneous implantation in mice, the modified cellulose was completely degraded within 20 days [27, 28].

Other approaches how to render the cellulose degradable, at least partially, is its oxidation and other chemical modifications of cellulose, such as its conversion into regenerated cellulose or 2,3-dialdehydecellulose. In addition, cellulose of animal origin, that is, from tunicates, degraded more quickly than plant cellulose. For example, when cellulose films from *Styela clava* were implanted subcutaneously into rats for 90 days, they lost almost 24% of their initial weight, while the films prepared from wood pulp cellulose lost only less than 10% (for a review, see [19]).

Schizophyllan is another β-glucan used for potential wound healing application. It is an extracellular β-1,3 beta-glucan with β-1,6 branching, produced by the fungus *Schizophyllum commune*. In blends with PVA, it was used for electrospinning of nanofibrous scaffolds, which provided a suitable growth support for human dermal fibroblast. In experimental wound models *in vivo*, schizophyllan attracted macrophages, necessary for the first physiological phase of wound healing, that is, inflammation. Schizophyllan and other 1,3-β-glucans also increased collagen deposition, cellularity, formation of granulation tissue, and vascularity at the wound site [29].

Other glucans used for fabrication of nanofibrous scaffolds for skin tissue engineering and wound healing include dextran, starch and pullulan. According to the type of their glycosidic bonds, these polysaccharides belong to α-glucans.

Dextran is a branched complex glucan, in which the D-glucose units are linked by α-1,6 glycosidic bonds with branches from α-1,3 linkages. Dextran is of microbial origin; it can be produced, for example, by some lactic acid bacteria from sucrose. Dextran was used as a component of nanofibrous polyurethane-based wound dressings, in which dextran promoted neovascularization of the wound site, and also served as carrier for β-estradiol, an endogenous estrogen, a potent anti-inflammatory

agent, and mitogen for keratinocytes. In addition, the presence of dextran made the polyurethane dressing softer, more flexible, more wettable, and well-adherent to the wound and promoted hemostatic activity of the dressing. *In vitro*, the presence of dextran and β-estradiol enhanced the proliferation of 3T3-L1 fibroblasts on the scaffolds [30].

Dextran was also used as component of a bilayer scaffold for skin tissue engineering. The upper part of the scaffolds was made of electrospun blend of poly (ε-caprolactone-*co*-lactide) and poloxamer (i.e., Pluronic), and the lower part was made of a hydrogel composed of dextran and gelatin without the addition of a chemical crosslinking agent. The lower dextran/gelatin hydrogel layer provided a highly swollen three-dimensional environment similar to extracellular matrix (ECM) of soft tissues. Both part of the scaffolds supported the growth of adiposetissue-derived stem cells; however, the number of these cells on the hydrogels decreased with increasing content of dextran [31].

Dextran is degradable by dextranases, enzymes hydrolyzing (1 → 6)-alpha-D-glycosidic linkages. This enzyme is produced mainly by bacterial and fungi, but it was also detected in animal and human tissues, namely liver and spleen. Therefore, dextran is often chosen for biomedical applications, particularly drug delivery, because it is slowly degradable in human organism. Dextran molecules with Mw higher than 40 kDa are sequestered in the liver and spleen, and then hydrolyzed by endo- and exodextranases. Dextran molecules with Mw lower than 40 kDa can be eliminated through renal clearance [32]. However, dextran hydrogels implanted subcutaneously or intramuscularly into rats did not show signs of degradation 6 weeks post-implantation and were surrounded by a thin fibrous capsule and some macrophages and giant cells, which is a response typical for a number of non-degradable materials [32].

Starch is another α-glucan, containing both α-1,4- and α-1,6 glycosidic bonds. It serves an energy storage polysaccharide in plants, and from this point of view, it is considered to be an analogue of glycogen, energy storage polysaccharide in animals. Starch consists of two types of molecules, namely linear amylose and branched amylopectin (for a review, see [33]). Electrospun starch-based nanofibrous meshes were proposed for wound healing applications. The electrospinning of starch was facilitated by addition of PVA, that is, a noncytotoxic, water-soluble, biocompatible synthetic polymer which reduced the repulsive forces produced in starch solution. The scaffolds then promoted the proliferation of mouse L929 fibroblasts [34]. Starch is degradable by amylases, that is, hydrolases that act on α-1,4-glycosidic bonds. Amylases occur in three forms, namely α-, β-, and γ-amylases. These enzymes are synthesized by microorganisms (bacteria and fungi), plants, and with exception of β-amylases, also in animals. Alpha-amylases are present in human organism, but not currently in all tissues-they are important enzymes of gastrointestinal tract and are produced by salivary glands and pancreas. Interestingly, α-amylases were also found in brain, and their lower expression there is probably associated with the pathogenesis of Alzheimer's neurodegenerative disease [35].

Pullulan is also an α-glucan with both α-1,4- and α-1,6 glycosidic bonds. It is a linear polysaccharide consisting of maltotriose units, in which three glucose units in maltotriose are connected by an α-1,4 glycosidic bond, whereas consecutive maltotriose units are connected to each other by an α-1,6 glycosidic bond. Pullulan is produced from starch by the fungus *Aureobasidium pullulans*. It shows a high water-absorbing capability, adhesive properties, and the capability to form strong resilient films and fibers. It is degradable by pullulanase, a specific kind of glucanase, produced in bacteria and not present in human tissues. When pullulan hydrogels alone or in combination with dextran were implanted subcutaneously into rats, they induced inflammatory reaction and were surrounded by a fibrous capsule [36]. Nevertheless, pullulan is water-soluble and thus removable from human issues, and

in combination with chitosan and tannic acid, it was used for fabrication of electrospun nanofibrous meshes promising for wound healing [37]. In combination with dextran and gelatin, pullulan was used for electrospinning of nanofibrous scaffolds promising for skin tissue engineering. These scaffolds, especially when crosslinked with trisodium trimetaphosphate, supported the adhesion and spreading of human dermal fibroblasts and formation of actin cytoskeleton in these cells [38].

Xylan is a plant polysaccharide belonging to the group of hemicelluloses, that is, polymers often associated with cellulose. While cellulose is made of glucose units, hemicelluloses contain many different sugar monomers. Xylans are polysaccharides made of β-1,4-linked xylose (i.e., a pentose sugar) residues with side branches of α-arabinofuranose and α-glucuronic acids, which contribute to crosslinking of cellulose microfibrils and lignin through ferulic acid residues. Xylans are considered as relatively available and cost-effective natural materials for tissue engineering. Electrospun nanofibers containing beech-derived xylan and PVA were tested as potential dermal substitutes for skin tissue regeneration. These scaffolds provided a good support for the adhesion and proliferation of human foreskin fibroblasts and for production of collagen by these cells [39]. Bagasse xylan was also a component of hydrogels endowed with shape memory, namely carboxymethyl xylan-g-poly(acrylic acid) hydrogels, applicable in tissue engineering and biosensorics, particularly for construction of electronic skin [40].

Alginates, for example, sodium alginate or calcium alginate, are salts of alginic acid, a linear polysaccharide composed of (1,4)-β-D-mannuronic acid and (1,3)-α-L-guluronic acid. Alginates are produced by various species of brown algae, and also by the bacterium *Pseudomonas aeruginosa*, a major pathogen found in the lungs of patients with cystic fibrosis. The structure of alginates is similar to glycosaminoglycans, an important component of ECM in human tissues including skin [41]. Alginates have a great ability to keep moisture in the wound site and to adhere to skin. However, alginates are poorly spinnable, and therefore, for skin tissue engineering and wound dressing applications, they were electrospun together with other polymers, such as PVA [41, 42] or PEO [43]. Poor mechanical properties of alginates have been compensated by the combination with chitosan [44] or PCL [44, 45]. In addition, alginates themselves are not adhesive for mammalian cells, which was compensated by their combination with collagen and gelatin, containing ligands for cell adhesion receptors [41]. Alginates were modified with a cell adhesive GRGDSP oligopeptide, which acts as ligand for integrin cell adhesion receptors [43]. Sodium alginate was used for attachment of arginine to the surface of chitosan nanofibers in order to increase healing capability of this wound dressing [46]. Alginate nanofibers supported by PCL were impregnated with an extract from *Spirulina*, a photosynthetic cyanobacterium producing bioactive molecules with anti-oxidant and anti-inflammatory effects [45]. Electrospun sodium alginate nanofibers containing silver nanoparticles were used for fabrication of an electronic skin capable of pressure sensing and endowed with antibacterial activity [47].

The degradability of alginate in human organism is limited. Alginate is naturally degraded by alginate lyases or alginate depolymerases, which have been isolated from marine algae, marine animals, bacteria, fungi, viruses, and other microorganisms, but are not present in the human organism. Degradability of alginate can be increased by its oxidation and at low pH. Also the hydrophilicity and water uptake capacity of alginate can help in its removal from the wound site (for a review, see [48]).

Pectin is a complex of structural polysaccharides present in the cell walls of terrestrial plants, rich in galacturonic acid. Pectin is known as gelling agent in food industry, but it is also widely used in medicine, for example, against digestive disorders, such as obstipation and diarrhea, for oral drug delivery, as a component of dietary fibers trapping cholesterol and carbohydrates, as a demulcent, that is, a

mucoprotective agent, and also in wound healing preparations [49]. Pectin is known as a natural prophylactic substance against poisoning with toxic cations, and its hemostatic and curing effects are well-documented in healing ointments [50]. Pectin is degradable by enzymes produced by bacterial, fungal and plant cells, and not present in human tissues [51–53]. Thus, pectin is degradable, at least partly, only in the intestinal tract populated with bacteria. However, pectin is water-soluble and quickly dissolves in the water environment, including the tissues. Therefore, in order to increase its stability, it was combined with chitosan and TiO_2 nanoparticles for wound dressing applications [50] or used for construction of composite chitosan-pectin scaffolds for skin tissue engineering. Blending chitosan with pectin markedly improved the mechanical properties of the scaffolds, such as their Young's modulus, strain at break and ultimate tensile strength, in comparison with pure chitosan scaffolds, although the proliferation of cells (i.e., fibroblasts) was slightly slower on pectin-containing scaffolds [54, 55]. The reason is that pectin does not contain cell binding domains. The cell adhesion on pectin nanofibers was markedly enhanced by oxidizing pectin with periodate to generate aldehyde groups, and then crosslinking the nanofibers with adipic acid dihydrazide to covalently connect pectin macromolecular chains with adipic acid dihydrazone linkers. In addition, the crosslinked pectin nanofibers exhibited excellent mechanical strength and enhanced body degradability [56].

Other polysaccharides explored for creation of nanofibrous scaffolds for skin tissue engineering and wound healing are gum tragacanth and gum arabic, both polysaccharides of plant origin, degradable by bacteria and fungi, for example, in soil [57, 58].

Gum tragacanth is a viscous water-soluble mixture of polysaccharides, mainly tragacanthin and bassorin. Tragacanthin dissolves to give a colloidal hydrosol. Bassorin, representing 60–70% of the gum, is insoluble and swells to a gel. Chemically, tragacanthin is a complex mixture of acidic polysaccharides containing D-galacturonic acid, D-galactose, L-fucose (6-deoyl-L-galactose), D-xylose, and L-arabinose. Bassorin is probably a methylated tragacanthin. A small amount of cellulose, starch, protein and ash are also present (https://colonygums.com/tragacanth). In order to improve electrospinning and mechanical properties of the gum tragacanth, it was combined with PVA and PCL [59]. Gum tragacanth is endowed with microbial resistance and wound healing activity, which was further enhanced by curcumin, a naturally occurring poly-phenolic compound with a broad range of favorable biological functions, including anti-cancer, anti-oxidant, anti-inflammatory, anti-infective, angiogenic, and healing properties [60].

Gum arabic, also known as gum acacia, is a complex and water-soluble mixture of glycoproteins and polysaccharides consisting mainly of arabinose and galactose. For skin tissue engineering, it was electrospun with PCL and also with zein, a storage plant protein [61].

Silk fibroin is a water-insoluble elastic protein present in silk fibers produced by larvae of *Bombyx mori* and some other moth of the *Saturniidae* family, such as *Antheraea assama, Antheraea mylitta*, and *Philosamia ricini* [62–64]. Silk fibroin occurs in the fibers together with sericin, a water-soluble serine-rich protein, which forms a glue-like layer coating two singular filaments of fibroin.

In biomaterial science, silk fibroin is considered to be degradable, but in mammalian organism, this degradation is long-lasting and can take more than 1 year. As a kind of biomaterial approved by the Food and Drug Administration (FDA) for medical use, silk is defined by United States Pharmacopeia as non-degradable for its negligible tensile strength loss *in vivo*. However, silk fibroin is susceptible to biological degradation by proteolytic enzymes such as chymotrypsin, actinase, carboxylase, proteases XIV, XXI and E, and collagenase IA. The final degradation products of silk fibroin are amino acids, which are easily absorbed *in vivo* (for a review, see [65]).

The degradation behavior of fibroin scaffolds depends on the preparation method and structural characteristics, such as processing condition, pore size, and silk fibroin concentration (for a review, see [65]). For example, three-dimensional porous scaffolds prepared from silk fibroin using all-aqueous process degraded within 2–6 months after implantation into muscle pouches of rats, while the scaffolds prepared using an organic solvent, hexafluoroisopropanol (HFIP), persisted beyond 1 year. It was probably due to a lower original silk fibroin concentration, larger pore size, and a higher and more homogeneous cellular infiltration of aqueous-derived scaffolds than in HFIP-derived scaffolds [66].

For skin tissue engineering and wound healing, silk fibroin has been combined with various synthetic and natural polymers and other bioactive substances. The polymers included, for example, PCL, [67], poly(L-lactic acid)-*co*-poly(ε-caprolactone) (PLACL, [68]), carboxyethyl chitosan, PVA, [69], chitin [70], cellulose-based materials modified by oxidation [71] or with lysozyme [72], collagen [73], gelatin [74], and hyaluronan [75]. The bioactive substances were, for example, growth factors, such as epidermal growth factor [64], vitamins, such as vitamin C [68], vitamin E [76], and pantothenic acid (vitamin B5; [77]), antioxidants, such as grape seed extract ([78]) or quinone-based chromenopyrazole [79], antibiotics, such as ciprofloxacin [64], tetracycline [68] or gentamycin [62], and other antimicrobial and wound healing agents, such as silver nanoparticles, dandelion leaf extract [63], *Aloe vera* [80], or astragaloside IV [74]. In order to enlarge the pore size in nanofibrous scaffolds for cell penetration, silk fibroin was electrospun together with so-called "sacrificial" crystals of ice [67] or NaCl [81, 82], that is, crystals which are removed after the electrospinning process. An interesting combination is silk fibroin with decellularized human amniotic membrane, which was used for developing a three-dimensional bi-layered scaffold for burn treatment. Adipose tissue-derived mesenchymal stem cells seeded on this scaffold increased expression of two main pro-angiogenesis factors, vascular endothelial growth factor, and basic fibroblast growth factor [83]. Also the transplantation of bone marrow-derived mesenchymal stem cells and epidermal stem cells into wounds using nanofibrous silk fibroin scaffolds supported re-epithelization, collagen synthesis, as well as the skin appendages regeneration [84]. Another interesting approach is to use silk fibroin produced by other species than *Bombyx mori*, namely by the moths *Antheraea assama* and *Philosamia ricini*. This "non-mulberry" silk fibroin possesses inherent Arg-Gly-Asp (RGD) motifs in its protein sequence, which facilitates binding of cells through their integrin adhesion receptors [64].

Sericin has also been applied in skin tissue engineering and wound healing, although in a lesser extent than silk fibroin. Sericin shows antioxidant, UV-protective, heat-protective, moisture-retaining, and antimicrobial properties, which have been reported to be more pronounced in non-mulberry sericin (e.g., from *Antheraea mylitta*) than in sericin produced by *Bombyx mori*. The reason is that wild moths like *Antheraea mylitta* are exposed to a hostile environment in nature than *Bombyx mori* raised in captive conditions. Similarly as non-mulberry silk fibroin, also sericin has been reported to be more supportive for cell adhesion than mulberry sericin (for a review, see [85]). Sericin enhanced the proliferation and epidermal differentiation of human mesenchymal stem cells on gelatin/hyaluronan/chondroitin sulfate nanofibrous scaffolds [86]. Similarly, sericin improved the growth of murine L929 fibroblasts and human HaCaT keratinocytes cultured on the PVA nanofibrous scaffolds [87] and also the growth of L929 fibroblasts on chitosan nanofibrous scaffolds, together with antibacterial properties of these scaffolds [88].

3. Nature-derived nanofibers degradable in the human tissues

Nature-derived polymers degradable in human tissues include, in particular, polymers that are synthesized in the human body and usually act as components of ECM. These polymers are proteins (collagen and its derivative gelatin, elastin, fibrinogen and fibrin, keratin) or polysaccharides in non-sulfated form (hyaluronic acid) and sulfated form (heparin-like glycosaminoglycans). In addition, some natural polymers synthesized by other organisms, such as bacteria, fungi, insects, crustaceans or plants, are degradable in human tissues, because they are susceptible to enzymes present in human tissues, such as lysozyme and esterases. These polymers include chitosan, gellan gum, zein, and PHBV.

Collagen is the main structural protein in the extracellular space in a wide range of tissues in the body. Skin contains type I collagen, one of the most abundant collagens in the human body. Type I collagen is also abundant in tendons, ligaments, and vasculature, and it is a main component of the organic part of bone. Type I collagen is a fibrillar type of collagen; it is composed of amino acid chains forming triple-helices of elongated fibrils. That is why the nanofibrous collagen scaffolds closely mimic the architecture of the native ECM and are advantageous for tissue engineering. In addition, collagen has been reported to be relatively poorly immunogenic, even if used in allogeneic and xenogeneic forms, for example, recombinant human collagen or bovine and porcine collagen. However, mammalian collagen is associated with the risk of disease transmission, for example, bovine spongiform encephalopathy (for a review, see [89–91]). This risk can be reduced by the use of fish collagen, which became to be popular in tissue engineering, including skin tissue engineering and wound healing. In addition, the fish collagen enables an easier recovery of intact collagen triple helices than the mammalian collagen [92]. Fish collagen can be obtained from the skin, scales and bones of freshwater fish, such as tilapia [91–94], and marine fish, such as hoki fish (*Macruronus novaezelandiae*) [92, 95], or *Arothron stellatus,* also known as "stellate puffer," "starry puffer" or "starry toadfish" [96]. Nanofiber electrospun from tilapia skin collagen promoted the proliferation of human HaCaT keratinocytes, and stimulated epidermal differentiation through the up-regulated gene expression of involucrin, filaggrin, and type I transglutaminase in these cells. Moreover, the tilapia collagen nanofibers accelerated wound healing *in vivo* in rat models [91–94]. Beneficial effects on wound healing were also observed in nanofibrous meshes electrospun from collagen obtained from *Arothron stellatus* [96] and from fish scale collagen peptides [90].

Collagen is one of the most widely used natural proteins for creation of nanofibrous scaffolds for skin tissue engineering and wound healing. However, these scaffolds are usually mechanically weak, and therefore they need crosslinking or blending with synthetic polymers. Collagen crosslinking with conventionally used agents, particularly glutaraldehyde, is associated with the risk of the scaffold cytotoxicity. More benign crosslinkers used recently include, for example, citric acid [95] or quaternary ammonium organosilane, a multifunctional crosslinking agent, which improved the electrospinnability of collagen by reducing its surface tension, endowed the collagen nanofibers with potent antimicrobial activity and promoted the adhesion and metabolic activity of primary human dermal fibroblasts without any cytotoxicity, at least in a lower concentration of 0.1% w/w [97].

Synthetic polymers used for combination with collagen in nanofibrous scaffolds included PLA [98], PLGA [99, 100], and particularly PCL, which was either blended with collagen [101–104] or served as substrate for subsequent deposition of collagen [105]. Collagen has also been combined with natural polymers, such as silk fibroin [73] or chitosan in a form of blends [106] or in a form of bilayered scaffolds, where collagen was electrospun onto the chitosan scaffolds [107]. Collagen was also

grafted on the surface of composite electrospun PVA/gelatin/alginate nanofibers [41]. Collagen-based or collagen-containing nanofibers have been loaded with a wide range of bioactive substances, such as vitamin C, vitamin D3, hydrocortisone, insulin, triiodothyronine, and epidermal growth factor [100], transforming growth factor-β1 [102], plant extracts such as *Coccinia grandis* leaf extract [96], or lithospermi radix extract [107], antibiotics such as gentamicin [103], or bioactive glass [93, 104]. Collagen and PCL and bioactive glass nanoparticles were applied for delivery of endothelial progenitor cells into wounds in order to promote their vascularization and healing [104].

Gelatin is a derivative of collagen, obtained by denaturing its triple helical structure. Specifically, it is a mixture of peptides and proteins produced by partial hydrolysis of collagen extracted from the skin, bone, and connective tissue of animals, such as cattle, pigs, chicken, and also fish. Gelatin can be defined as a complex mixtures of oligomers of the α subunits joined by covalent bonds, and intact and partially hydrolyzed α-chains of varying molecular weight (for a review, see [89, 92]). Properties of gelatin, including its spinnability, depend on the source of collagen, animal species, age, type of collagen, type of conversion of collagen to gelatin (i.e., acidic *vs.* basic hydrolysis), and particularly on the conformation of gelatin molecules [108].

Similarly as collagen, also gelatin is the most promising for skin tissue engineering and wound healing applications in combination with various synthetic and natural polymers. For example, gelatin was combined with polyurethane [109], PLA [11, 17], and particularly with PCL, where it was incorporated into core-shell PCL/gelatin nanofibers as the core polymer [110] or electrospun independently of PCL using a double-nozzle technique, which resulted in creation of two types of nanofibers in the scaffolds, either mixed [111] or arranged in separate gelatin and PCL layers [112]. Gelatin was also combined with a copolymer of lactic acid and caprolactone P(LLA-CL) in the form of blends [113] or in the form of coaxial nanofibers with P(LLA-CL)/gelatin shell and albumin core containing epidermal growth factor, insulin, hydrocortisone, and retinoic acid [114]. Natural proteins combined with gelatin included dextran [31], pullulan [38], alginate [41], silk fibroin [74], and hyaluronan with chondroitin sulfate [86].

For combination with synthetic and natural polymers, for example, with PCL [115], and chitosan and keratin [116], gelatin was also used in the form of photocrosslinkable gelatin methacrylate hydrogel (GelMA). On PCL nanofibers, GelMA showed a higher decoration level in comparison with native gelatin [116]. Self-standing nanofibrous matrices electrospun from GelMA enabled tuning of their water retention capacity, stiffness, strength, elasticity, and degradation by changing the exposure time to UV light [117].

Elastin is a protein found in the ECM, that maintains its elasticity [118]. It is the second main protein-based component of native skin ECM. The presence of elastin in composite electrospun nanofibrous scaffolds, containing gelatin, cellulose acetate and elastin, changed the fiber morphology from straight to ribbon-like structure, and decreased the swelling ratio and degradation rate of the scaffolds. In addition, elastin-containing scaffolds supported the attachment and proliferation of human fibroblasts [119].

Fibrin is a provisional ECM protein, which accumulates in wounds after injury to initiate hemostasis and healing. Fibrin is formed via the polymerization of fibrinogen monomers in the presence of thrombin, and this process can be simulated *in vitro* [120]. Fibrin forms a fine nanofibrous mesh, which is mechanically weak and needs to be deposited on some supportive structure, for example, synthetic nanofibrous meshes made of poly(L-lactide) (PLLA) [121]. In our experiments, fibrin was deposited on PLLA in the form of two types of coating, depending on the mode of

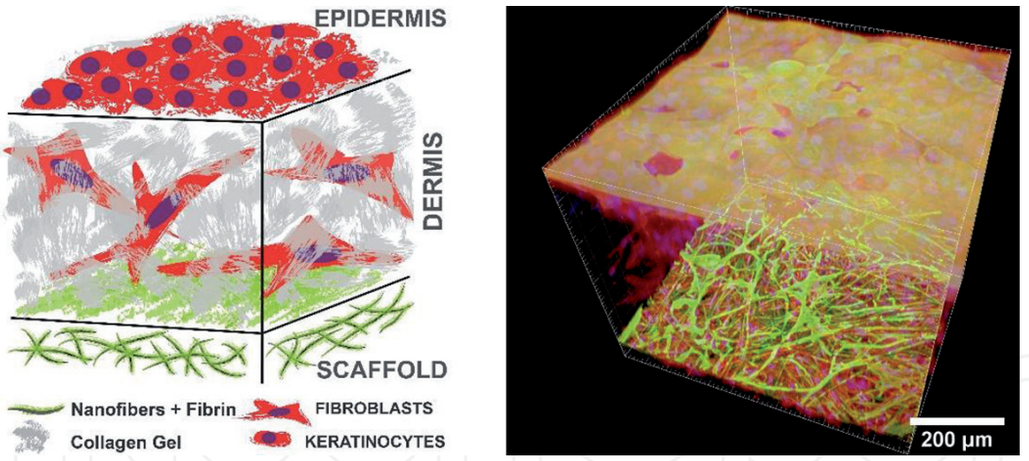

Figure 2.
Developing a bilayer construct of keratinocytes and fibroblasts on a PLLA nanofibrous membrane with fibrin and collagen hydrogel. Left: schematic design; right: real construct.

fibrin preparation. Fibrin either covered the individual fibers in the membrane (F1 nanocoating), or covered the individual fibers and also formed a fine homogeneous nanofibrous mesh on the surface of the membrane (F2 nanocoating), depending on the mode of fibrin preparation. The fibroblasts on the F1 nanocoating remained in their typical spindle-like shape, while the cells on the F2 nanocoating were polygonal with a higher proliferation rate [122]. F2 nanocoating was then used for development of a bilayer skin construct. First, a nanofibrous PLLA mesh was coated with fibrin and seeded with human dermal fibroblasts. After reaching confluence, the fibroblasts were covered with a collagen hydrogel and were allowed to migrate into this hydrogel and to proliferate inside. After sufficient colonization of the hydrogel with fibroblasts and formation of a structure resembling the skin dermis, human epidermal keratinocytes were seeded on the top of the collagen hydrogel (**Figure 2**) [123].

Also fibrinogen was used for modification of synthetic polymeric nanofibers in order to enhance the cell adhesion and growth. Nanofibrous scaffolds electrospun from blends of PCL and fibrinogen improved the adhesion, proliferation, and epidermal differentiation of adipose tissue-derived stem cells (ADSCs) in comparison with pure PCL scaffolds. Composite PCL/fibrinogen scaffolds seeded with ADSCs also markedly improved healing of full-thickness excisional wounds created in rats in comparison with acellular dermal matrix or acellular dermal matrix with ADSCs [124].

Keratin is a fibrous structural protein, present in skin appendages, such as hair, wool, feather, nails, horns, claws, hooves, and in the outer (cornified) layer of epidermis [125, 126]. Keratin protects epithelial cells from damage and stress and is insoluble in water and organic solvents.

In most studies dealing with keratin-containing nanofibers, keratin was combined with other natural or synthetic polymers in order to improve the spinnability of keratin, or to improve the bioactivity of the co-electrospun polymer. For example, in a study by Cruz-Maya *et al*. [127], blending keratin with PCL improved the stability of the electrospinning process, promoted the formation of nanofibers without defects, such as beads and ribbons, typically observed in the fabrication of keratin nanofibers. At the same time, keratin markedly increases the fiber hydrophilicity compared with pure PCL, which improved the adhesion and proliferation of human mesenchymal stem cells [127]. Similarly, co-electrospinning of keratose (i.e., oxidative keratin) with PVA resulted in nanofibers with uniform fibrous structure, suitable hydrophilicity and mechanical properties [125]. Properties of electrospun keratin nanofibers were also improved by incorporation of hydrotalcites, intended for delivery of diclofenac. These nanofibers displayed a reduced swelling

ratio and a slower degradation profile compared to keratin-based non-woven nanofibrous mats containing free diclofenac [126]. Keratin was also a component of core-shell nanofibers, prepared by coaxial electrospinning of chitosan, PCL and keratin with *Aloe vera* extracts encapsulated inside the polymer nanofibers. This construct increased the adhesion and growth of L929 fibroblasts and was intended for wound healing applications [128]. Importantly, keratin was a component of bilayer scaffolds for skin tissue engineering, composed of human hair keratin/chitosan nanofiber mat and gelatin methacrylate (GelMA) hydrogel. Human dermal fibroblasts were encapsulated and grown in the hydrogel matrix, while human HaCaT keratinocytes formed a layer on the top of the scaffolds, mimicking dermis and epidermis of skin tissue [116]. Another bilayer scaffolds was constructed using polyurethane wound dressing as an outer layer, and electrospun gelatin/keratin nanofibrous mat as an inner layer [109].

Hyaluronic acid, also called hyaluronan, is an anionic, non-sulfated linear glycosaminoglycan. It is distributed widely throughout connective, neural, and epithelial tissues, including skin, where it is a major component of ECM. Therefore, hyaluronic acid has been widely used for skin tissue engineering and wound healing, and it is approved for clinical application [33].

Hyaluronic acid stimulated infiltration of nanofibrous scaffold composed of hyaluronan, silk fibroin and PCL [75], and can help to promote cell proliferation [129]. Electrospinning of pure hyaluronic acid is not simple because of solubility characteristics of this polymer. Hyaluronic acid is well-soluble in water but less-soluble in most organic solvents, which can be solved by mixtures of solvents as water/ethanol or water/dimethylformamide [130]. Increasing of evaporation and decreasing of solution surface tension by the solvent mixing helps to electrospinning process. Another possibility is electrospinning of hyaluronic acid together with a suitable water-soluble polymer such as PVA [131] or PEO. The solution of pure hyaluronic acid [132] or with relatively small amount of carrier PEO was successfully spun into nanofibrous material by air-assisted electrospinning technology, that is, electroblowing [133]. For creation of nanofibrous scaffolds, hyaluronic acid was also used in combination with PCL [134], PLA [135] or gelatin, chondroitin sulfate and sericin [86].

Sulfated glycosaminoglycans have been relatively rarely used as components of nanofibers for skin tissue engineering and wound healing in comparison with hyaluronic acid. This group of polysaccharides include heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate (for a review, see [33]). From these polysaccharides, only heparin, heparan sulfate, and chondroitin sulfate were used as components of nanofibers for skin regenerative therapies. For example, heparin coatings on PLLA nanofibers increased the infiltration of the scaffolds with endothelial cells *in vitro,* and enhanced epidermal skin cell migration across the wound in a full-thickness dermal wound model in rats *in vivo* [136]. In a recent study by Yergoz *et al*. [137], a heparin-like nanofibrous hydrogel promoted regeneration of full thickness burn injury in mice. Chondroitin sulfate was used as a component of electrospun gelatin/PVA/chondroitin sulfate nanofibrous scaffolds, which supported the proliferation of human dermal fibroblasts [138], of electrospun nanofibrous composite scaffolds made of cationic gelatin/hyaluronan/chondroitin sulfate loaded with sericin, which promoted the differentiation of human mesenchymal stem cells toward epithelial lineage [86], and of electrospun gelatin/chondroitin sulfate nanofibrous scaffolds, which accelerated healing of full-thickness skin excision wounds in rats [139].

Chitosan is a linear polysaccharide composed of randomly distributed β-(1 → 4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine

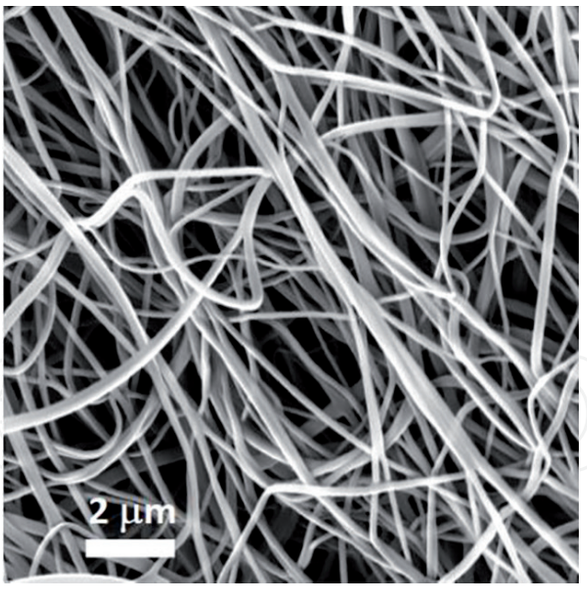

Figure 3.
Scanning electron microscopy of nanofibrous layers produced by needle electrospinning from PVA/chitosan solution.

(acetylated unit). It can be prepared by alkali treatment from chitin, a poly-N-acetyl-D-glucosamine polysaccharide, the major structural component of the exoskeleton of crustaceans (crab, shrimp), and of the cell wall of fungi and yeast [140]. Chitosan is known as biocompatible, antimicrobial, and biodegradable. In the human organism, it can be degraded by lysozyme, a hydrolytic enzyme present in various secretions such as saliva, tears, mucus, and human milk, and also in cytoplasmic granules of macrophages and polymorphonuclear neutrophils. Chitosan breakdown by lysozymes happens via the removal of glycosidic bonds between polysaccharide units in the polymer. Glucosamine and saccharide are the products of this process, which can be metabolized or stored as proteoglycans in the body.

However, electrospinning of chitosan is difficult due to its polycationic characters. Due to the presence of amine groups in the chitosan molecule, acidic aqueous solutions are the best solvents for this polymer. The best candidates for solvent system seem to be mixture of acetic acid (AA) and formic acid (FA) or trifluoroacetic acid (TFA); however, TFA is highly toxic. Electrospinning of pure chitosan has very low productivity because it requires very concentrated polymeric solutions [141]. Therefore, for creation of nanofibrous scaffolds for skin tissue engineering, chitosan has been mixed with other natural or synthetic polymers, such as collagen [142], gelatin [143], keratin [116], cellulose [144], pectin [54, 55], silk fibroin [69], PHBV [145], PCL [142], PLA [146], PLGA [147], PEO, [148], and also with PVA, which was used in our studies (**Figure 3**). Chitosan has also been mixed with various nanoparticles, such as halloysite nanotubes [149], graphene oxide [150] or nanodiamonds [144]. The reason of all these mixtures was to improve the stability, spinnability, wettability, mechanical properties, and biofunctionality of chitosan-containing scaffolds for skin tissue engineering. Combination of chitosan with various polymers also enabled creation of bilayer scaffolds for reconstruction of two main skin layers, that is, epidermis containing keratinocytes and dermis containing fibroblasts [116, 142]. In order to enhance the antimicrobial and wound healing activity of chitosan, this polymer was electrospun together with extract from Henna leaves [151]. In addition, chitosan nanoparticles have been incorporated in nanofibrous scaffolds as carriers for controlled drug delivery, for example, delivery of growth factors, cytokines and angiogenic factors, such as platelet-derived growth factor [152], granulocyte colony-stimulating factor [153] or angiogenin [147].

Nanofibrous scaffolds promising for skin tissue engineering and wound healing were also prepared directly from chitin, which was electrospun either alone with further modifications with fibronectin, laminin and particularly with type I collagen [154], or in combination with silk fibroin [70].

Gellan gum is a water-soluble anionic polysaccharide produced by the bacterium *Sphingomonas elodea* (formerly *Pseudomonas elodea*). The repeating unit of the polymer is a tetrasaccharide, which consists of two residues of D-glucose, one of residue of L-rhamnose and one residue of D-glucuronic acid. For skin tissue engineering and wound healing, gellan gum was electrospun with PVA in order to decrease its viscosity and repulsive forces between the polyanions along the polymer chains and to increase the stability, uniformity, and structural consistency of the nanofibers in aqueous environment. The nanofibrous scaffolds were further stabilized by crosslinking with various physical, chemical, and ionic methods, such as by heat, UV irradiation, methanol, glutaraldehyde, and by calcium chloride [155]. These scaffolds supported the adhesion and growth of human dermal 3T3-L1 fibroblasts [155, 156] and human HaCaT keratinocytes [157] and provided a better support for these cells than conventionally proposed gellan-based hydrogels and dry films. In addition, these scaffolds were endowed with antimicrobial activity by incorporation with amoxicillin, and accelerated healing of full-thickness skin excision wound in rats in comparison with non-treated wounds [157]. Similarly as chitosan, gellan gum has been reported to be degradable by lysozyme [158]. Three-dimensional printed gellan gum scaffolds also showed degradation *in vitro* in phosphate-buffered saline (PBS) or in simulated body fluid, and the degradation rate could be modulated by changing the ratio of surface area per mass of the scaffolds [159].

Zein is the major storage protein of corn, composed of amino acids such as leucine, glutamic acid, alanine and proline, and showing good biocompatibility, flexibility, microbial resistance, and antioxidant activity [61]. Zein has been shown to degradable *in vitro* in PBS and also *in vivo* when implanted subcutaneously in rats in the form of rod-like implants [160]. However, similarly as in many other natural polymers, the application of pure zein nanofibers is limited because of poor mechanical properties of these fibers. Therefore, for skin tissue engineering and wound dressing applications, zein has been mixed with various synthetic and nature-derived polymers, such as polyurethane [161], PLA [162], PCL, hyaluronic acid, chitosan [163], and polydopamine [164], and impregnated with TiO_2 nanoparticles [164] or Ag nanoparticles [161] in order to enhance the antimicrobial activity of the scaffolds.

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), is a polymer produced naturally by bacteria as a storage compound under growth-limiting conditions. It is a thermoplastic linear aliphatic polyester of polyhydroxyalkanoate type. PHBV is approved by the FDA for medical use. PHBV is biodegradable by bacterial enzymes, but it is also susceptible to hydrolytic degradation in water environment, although this degradation is relatively slow. When degradation of porous PHBV scaffolds for tissue engineering was simulated *in vitro* in PBS at 37°C, it lasted several months [165]. In the human body, however, the degradation of PHBV can be accelerated by nonspecific esterase and lysozyme enzymes, both present in cells of the immune system (for a review, see [165]). For biomedical application, PHBV is often used as an alternative to synthetic polymers, but it has several drawbacks, such as relatively high cost, brittleness, relatively difficult processing, and also hydrophobicity, which can hamper the cell adhesion and growth. However, PHBV is piezoelectric, which can stimulate the adhesion, growth, and phenotypic maturation of cells. Pure electrospun PHBV meshes supported the adhesion, growth, and epidermal differentiation of bone marrow mesenchymal stem cells, which was induced by an appropriate composition of cell culture media, containing epidermal

growth factor, insulin, 3,3′,5-triiodo-L-thyronine (T3), hydrocortisone, and 1α, 25-dihydroxyvitamin (D3), and manifested by expression of genes for keratin, filaggrin, and involucrin, that is, an early, intermediate and late marker of keratinocyte differentiation, respectively [166]. In order to increase the attractiveness of electrospun PHBV nanofibers for the cell adhesion and growth, they were coated with collagen [167] blended with collagen [168], blended with chitosan [145] or blended with keratin [169]. Collagen-coated PHBV nanofibers alone or seeded with unrestricted somatic stem cells, isolated from umbilical cord, accelerated closure of excision wounds in rats *in vivo* compared to unmodified PHBV nanofibers [167]. Similar wound healing effect was also obtained with PHBV nanofibers blended with keratin [169]. Mechanical properties of PHBV nanofibers were improved by addition of graphene oxide nanoparticles in the electrospinning solution, which also endowed these fibers with and antimicrobial activity [167].

4. Conclusions

Nanofibrous scaffolds made of nature-derived polymers hold a great promise for skin tissue engineering and wound healing. These scaffolds are created from biological matrices, and from this point of view, they resemble the extracellular matrix more closely than synthetic polymers. Some of these polymers, such as collagen, gelatin, elastin, keratin, nonsulfated and sulfated glycosaminoglycans, and also nonmulberry silk fibroin, contain motifs that are recognized and bound by cell adhesion receptors. Therefore, nature-derived polymers can increase the bioactivity of synthetic polymers, when combined with them in nanofibrous scaffolds. Conversely, synthetic polymers can improve the electrospinnability and mechanical properties of the natural polymers. Similarly as synthetic polymers, nature-derived polymers can be more or less degradable in human tissues. Degradable polymers include collagen, gelatin, elastin, keratin, glycosaminoglycans, but also chitosan, gellan gum and PHBV, that is, polymers produced by other than mammalian organisms. Polymers produced by other organisms, such as bacteria, fungi, algae, plants or insects, are usually nondegradable in human tissues, or their degradability is limited due to lack of appropriate enzymes. These polymers include glucans, such as cellulose or dextran, and other polysaccharides and proteins, such as pullulan, alginate, pectin, and silk fibroin. Well-degradable polymers are recommended as direct scaffolds for tissue engineering, while less-degradable polymers are suitable for "intelligent" wound dressing for drug delivery and cell delivery.

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