Designing multigradient biomaterials for skin regeneration

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Skin defects are amongst the main causes of morbidity and mortality worldwide, which account for significantly high socioeconomic costs. Today, much attention is being paid to tissue engineering and biomaterials strategies for skin regeneration, and among them, there is increasing interest in using multigradient biomaterials. Gradient-based approaches are an emerging trend in tissue engineering for the homogeneous delivery of therapeutic agents by using biomaterials. Several studies have acknowledged that wound repair mechanisms could be enhanced through biomimicking physicochemical properties of different skin layers. In addition, in different layers of skin tissue, cells experience various physicochemical gradients, which potentially regulate their behaviors. Therefore, interface tissue engineering and biomaterials approaches are gaining increasing attention for skin regeneration through the incorporation of physicochemical gradients within the engineered constructs. This review first presents a necessary overview of the biological properties of skin tissue and its changes during repair in different tissue injuries. Fundamental issues and necessities of using different types of gradient scaffolds and interface tissue engineering strategies for skin regeneration are addressed. The focus of this review is on describing current progress in designing gradient scaffolds for controlling and directing cellular and molecular responses in skin tissue. The main used fabrication approaches, including both traditional and advanced methods for designing multigradient scaffolds, are also discussed.

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1. Introduction

Skin wounds are among the most common devastating injuries, which cause suffering in millions of people worldwide [1–3]. Over the past few decades, biomaterials and interface tissue engineering fields have emerged with the aim of achieving appropriate treatments to overcome such devastating injuries [4,5]. The emergence of these fields results in suggesting many promising treatments for skin regeneration by combining biomaterials, cells, and therapeutic agents in different ways [6]. These strategies have been able to enhance wound healing by increasing the protection against dehydration and infection, as well as transporting and/or attracting the matrix components into the wound site [7–9]. In addition, functional mechanisms of biomaterials could aid in improving the innate wound healing processes, including the modulation of local inflammation, selective cell infiltration, and neovascularization [7]. According to traditional classifications, skin scaffolds could be categorized into three main groups based on (i) their structures (e.g. porous, fibrous, decellularized, hydrogels, and microspheres), (ii) type of biomaterials (e.g. natural, synthetic, and composites)
and (iii) different injured layers, including epidermal, dermal, and epidermal/dermal scaffolds [10–15]. However, it is a well-known fact that skin tissue is rich in multiple physicochemical gradients, which exist throughout the tissue and at its interface to satisfy various functional requirements [16,17]. Hence, compared to the above-mentioned traditional approaches for designing biomaterials, gradient-based strategies have currently gained substantial attention as more promising candidates in mimicking the skin tissue environment and also directing cellular and molecular pathways [18,19]. Gradient-based strategies could be successfully combined with the present interface tissue engineering approaches to engineer complex tissues [20]. Multigradient-based scaffolds have been able to offer a quick single-experiment way to enhance biomaterials’ functions without producing experimental artefacts [21]. In this manner, different physicochemical gradients could be directly combined during the biomaterial synthesis [21]. In fact, the combination of spatially controlled signal gradients with already existing skin regeneration approaches could lead to designing dynamic cellular scaffolds that are able to improve wound healing processes [20–23]. Here, we first give a concise overview of the natural skin tissue biology and its changes during wound healing processes. The principles of using multigradient scaffolds and interface tissue engineering strategies for skin regeneration are addressed. The focus of this paper is on discussing the current trend and the possibility of synthesizing multigradient scaffolds for skin regeneration applications.

2. The hierarchical composition of skin

All tissues, including skin, are rich in various physicochemical gradients, which have substantial effects on the cellular and molecular pathways [20]. The in vivo physical gradients, which could be detected throughout the skin and at its interface with other tissues, are mainly defined as the slow changes of different physical characteristics, such as stiffness, porosity, and topology [23]. In addition, two types of chemical gradients, including matrix-integrated and soluble biomolecules, exist throughout different skin layers [24,25]. Skin tissue is the first line of defense against the environment, an immunologically active sensory and excretory tissue that helps in regulating the body temperature [26]. Epidermis, dermis, and the underlying hypodermis are the three main layers of skin that are designed from two different types of tissues, including epithelium and connective tissues. Based on the density of keratinocytes and their cell-junction complexes, various layers could also be detected in epidermis, including the stratum corneum (SC), stratum lucidum (SL), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB) (Fig. 1). The protein-rich keratinocytes are mostly composed of keratin and keratohyalin, of which different patterns and concentrations could be detected in epidermis layers [25]. The SC is defined as a heterogeneous arrangement similar to “bricks and mortar” with corneocytes as hydrophilic “bricks” and intercellular lipid matrix as hydrophobic “mortar.” This layer contains a gradient of corneocytes with different thicknesses based on the anatomic site [27]. This layer could also be separated into two different layers, including stratum disjunctum and stratum compactum [28]. The stratum compactum has solid intercellular components, which make this layer the key diffusion barrier. In this layer, cells are connected together through corneodesmosomes, which are the key players in maintaining the SC cohesion. In addition, in the intercellular space, the SC lies in the lipid lamellae matrix that supports in preventing the loss of internal water [29]. Lamellar bodies, which secrete the precursor lipids in the SS and SG, are contrarily dependent on the Ca²⁺ concentration gradient so that a high amount of Ca²⁺ in the SB keeps the lamellar bodies' secretion at slow rates [24,25]. However, the upper layers of epidermis are rich in lipid lamellae, and the SB is rich in phospholipids [30]. SB is a noncellular protein-polysaccharide rich layer containing particular fibrils, PGs, and GPs (such as collagen type IV and VII, laminin). It should be noted that the entactin/nidogen complex, which is a key player in stimulating laminin/IV collagen complex and basal lamina self-assembly, is highly dependent on Ca²⁺ ion concentrations. Below this layer, the reticular lamina, with its high concentrations of collagen type III, is found. While the epidermis layers are rich in cells, the dermis layers are mostly composed of collagen and elastic fibers in a glycosaminoglycans’ (GAGs) gel [31]. The Dermis is composed of both loose and dense irregular connective tissue layers. The loose connective tissue layer is located just beneath the epithelium layers and composed of thin and sparse cells with a high concentration of the ground substance, as well as different inflammatory and immune cells. The dense irregular connective layer (reticular layer of dermis) contains low cell densities, typically single type-fibroblasts, with high collagen concentration. However, the hypodermis is rich in fat, sensory nerves, and GAGs, and connects the skin tissue to the inner organs. This layer supports insulation, protective padding, and energy storage. In general, in different epidermis layers, a specific cell–cell adhesion gradient (e.g. occluding, anchoring, and communicating junctions) could be seen, which is because, in the upper layers, keratinocytes are totally packed; however, in the lower layers, there is more space between them (see Fig. 1) [32]. Hence, a gradient of different necessary channels for paracellular pathways could be detected in different skin layers. In addition, because of the Ca²⁺ dependent manner of some cell-junctions complexes (such as E-cadherin/catenin and entactin/nidogen), a Ca²⁺ concentration gradient could also be seen in some layers [32]. Furthermore, in different layers of skin tissue, multiple extracellular matrix (ECM) components’ (such as collagen, PGs, GAGs) gradients could be expected in both their types and concentration aspects. A hypoxia gradient, which consequently affects the hypoxia-inducible factor 1-alpha, vascular endothelial growth factor and nitric oxide synthases (HIF-α, VEGF, and NOS), migration, and concentration have been also observed in the different skin layers [33–35].

3. Biological changes during wound healing processes

In general, skin wounds can be categorized into two acute and chronic types based on their healing time [36]. These injuries could be caused because of genetic irregularities, pathological processes, infections, irradiation, burns, and physical or chemical traumas.
Clinically, there are four main wounds based on the injury's depth, including epidermal, superficial partial-thickness, deep partial-thickness, and full-thickness skin wounds [37]. The first two types are commonly repaired through tissue self-healing functions. However, in deep partial-thickness and full-thickness skin wounds, the epithelial regenerative components are devastated, and self-healing cannot be done [38]. Wound healing is a biological complex process in which the damaged skin layers are repaired through several cellular and molecular pathways, including interactions among the ECM components, cells, cytokines, and growth factors [39]. Hemostasis, inflammation, proliferation, and remodeling are the four main, continuous, and occasionally overlapping stages of wound healing that should happen in the stated order and at the right time [40]. The hemostasis phase, which is mainly mediated by platelets, occurs instantly after the skin injury. In this phase, fibrinogens are transformed into fibrins, which is consequently the cause for stopping bleeding and forming a provisional fibrous matrix for subsequent cell migration. In addition, platelets, in a gradient manner release various cytokines and growth factors, such as transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), VEGF, and epidermal growth factor (EGF) [41]. After that, cytokines and growth factors provoke neutrophils, macrophages, and lymphocytes migration to the injured layers for starting the inflammation stage. The inflammatory cells enhance the blood circulation, as well as the expression of some pro-inflammatory factors, such as tumor necrosis factor (TNF) and colony-stimulating factor-1 (CSF-1). These factors subsequently improve the proliferation and migration of fibroblasts, endothelial cells, smooth muscle cells, and keratinocytes [42]. In addition, collagen fabrication will be stimulated by fibroblasts [43,44]. The inflammatory phase starts making the injured tissue ready for repair through reestablishing homeostasis and developing a barrier against microorganisms invasion [36,45]. Furthermore, phagocytes, in this stage, rush into the injured area and surround the dead cells [46]. The growth of granulation tissue is the main sign of the third stage known as proliferation, which causes re-epithelialization, fibroplasia, as well as angiogenesis. This generally occurs two days to three weeks after injury. It has been reported that oxygen gradients play pivotal roles in the three mentioned wound healing phases and could potentially regulate the inflammatory cells activation, fibroblast migration, collagen synthesis, and angiogenesis [47]. In the remodeling phase, changes in both ECM composition due to the reformation of collagen fibers type III to I and their orientation enhance the tissue mechanical strength. In fact, the transition from the granulation tissue to scar formation occurs during this phase [48]. This phase commonly starts three weeks after damage and can last up to two years. The wound healing process could only be successfully done when all the four-mentioned phases are occurring at the proper time, which is highly dependent on the skin injury type. For instance, in chronic wounds, a prolonged inflammatory phase could be detected, through which the over-activity of proinflammatory factors led to ECM degradation. However, in acute skin injuries, the remodeling phase is disturbed owing to unsuccessful myofibroblast apoptosis at a specific time. Thus, the specific injury type should be taken into consideration in designing scaffolds [48].

4. Essential considerations in designing scaffolds for skin regeneration

Over the past few decades, using different types of biomaterials has been considered as one of the best choices for skin regeneration. The American National Institute of Health has described a biomaterial as “any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ, or function of the body, in order to maintain or improve the quality of life of the individual” [49]. In general, a set of key requirements should be taken into account at the time of designing scaffolds for directing tissue growth, including their biocompatibility, bioactivity, (bio)degradability, physicochemical properties, and economic aspects [50,51]. Because of the existing physicochemical gradients in skin layers, considering some other design parameters for skin scaffolds is essential. Based on the features of two main layers of skin tissue (epidermis and dermis), several types of scaffolds have been suggested as multilayered scaffolds [52–54]. However, at the time of choosing a scaffold composition, various specific physicochemical continuous and non-continuous gradients in different layers of epidermis and dermis are also important. For instance, structural gradients are an important parameter of the scaffold, which should be designed so that it could provide a suitable niche for different skin cells to promote the new tissue formation, remodeling, vascularization, and integration. Additionally, the scaffold structure should be both porous and stable to provide both the diffusion of nutrients and metabolites [55]. The pore size of the scaffold is another key point, as it influences cell interactions with the scaffold and cell migration [56]. However, as in skin tissue, different types of cells could be found in its layers, the specific pore size suitable for each cell type should be considered. For instance, epidermis encompasses keratinocytes (95%), Langerhans, melanocytes, Merkel-Ranvier cells (tactile epithelial cells), and inflammatory cells; however, dermis contains fibroblasts, macrophages, and adipocytes, as well as several vessels' and nerve receptors [25]. Overall, for skin regeneration, 5 μm pore size could be suitable for neo-vascularization, 5–15 μm for fibroblast ingrowth, and 20–125 μm for dermal repair [57–60]. Additionally, the migration rates of skin cells is another key point. Fibroblasts can migrate up to 200 μm/day; however, they only produce ECM if the blood vessels and nutrients are within 100 μm. Hence, angiogenesis can be seen as a rate-limiting step [59,61]. The stiffness property of scaffolds and its specific changes throughout the tissue, which have substantial influences on the scaffold's degradation, and subsequently, tissue regeneration are also highly addressed [62]. Since the mechanical properties and scaffold architecture have direct influences on each other, a fine balance between them allows the scaffold to enhance satisfactory infiltration and vascularization, as well as correct stability upon implantation [55]. Having a specific gradient of different growth factors that play key roles during wound healing is also vital.

5. Gradients in designing scaffolds for skin regeneration

Several natural- and synthetic-based biomaterials have currently been suggested for skin regeneration applications and reviewed elsewhere [4,18,63]. This paper is limited in scope to the review of multigradient scaffolds that have been specifically synthesized based on the various physicochemical properties of skin layers.

5.1. Physical gradients

In different layers of skin tissue, substantial differences between physical properties, such as layer's stiffness, architecture, and composition could be observed, which should be taken into account at the time of selecting and designing biomaterials. Here we mention some of these aspects, and then in the next sections, some of the most current studies that have designed gradient skin scaffolds are mentioned. Regarding composition gradients, epidermis and dermis contain different sub-layers, which are formed and
adhered together through various natural biomaterials. The sub-layers of epidermis layer, as an epithelium tissue, have different physicochemical gradients in both continuous and noncontinuous ways. From top to the bottom of epidermis, the complexes responsible for inducing cell-cell junctions (such as occluding-claudin and E-cadherin/catenin) have different mechanisms of working with specific cellular signaling pathways. Each of these complexes works through using some specific ions and proteins (such as Ca\(^{2+}\) ions in E-cadherin/l-catenin and entactin/nidogen complexes), which could be used in scaffolds for stimulating the specific signaling pathways. In addition, in a skin, a gradient of different collagen types is detected from top to bottom. For instance, in the basement membrane, reticular lamina, loose and dense irregular connective tissue layers IV, VII, III, I collagens types in different concentrations, and orientations are seen, and each of them is responsible for providing a particular feature. The dermis layer is designed from both thin, loose, and thick, dense irregular connective tissue layers with a continuous gradient of GAGs and collagen (in different orientations) [64].

Furthermore, the stiffness gradient between different layers of the native skin induces the stress transmission to the fundamental mechanoreceptors for improving tactile sensing (Fig. 3a) [16]. Hence, for being able to do normal functions, the skin cells should adhere to a solid scaffold with special stiffness, which is characterized by its elastic modulus [25, 30, 65]. Cells are able to sense the biomaterial’s stiffness by applying forces via adhesion components and their actin-myosin cytoskeleton. Hence, it is undeniable that this mechanical property of biomaterials could have vital influences on directing cells’ functions, such as migration, differentiation, and proliferation through a mechanism named durotaxis or mechanotaxis [66]. Based on these mechanotransduction mechanisms, in multilayered tissues like skin, a stiffness gradient in different layers is expected, which could highly affect the biomaterial’s degradation and tissue regeneration processes [62]. However, the potential effects of scaffold’s stiffness on different cell types, such as fibroblast and epithelial cells, have been reported [62]: most of the current studies use homogeneous and/or step-gradient scaffolds by a lack rationale in choosing the stiffness amounts [20–23]. In addition, many of the existing suggested scaffolds for skin are in either soft or stiff categories; however, the native tissue experiences a continuous mild stiffness gradient. This kind of research does not provide useful data concerning the threshold amounts of stiffness, which could change the cellular behaviors at tissue interfaces. Moreover, an operative stiffness range differs between various cell types, which leads to particular cell responses in skin tissue with different cell types in each layer. Bearing in mind some systematical investigations for designing different types of scaffolds comprising incessant stiffness gradients in a wide range could help in providing data regarding skin cells’ responses to various stiffness amounts and finding the critical amounts at the interfaces [20, 22, 23].

Pore size and porosity are also among the critical properties for designed scaffolds based on the skin tissue structure [67, 68]. Pore size could have influences on cellular functions by affecting molecular and cellular migration, binding and spreading, signaling pathways, as well as nutrients’ transport [23]. The cells’ migration could be highly directed by reducing or increasing the pore size [21, 69]. The scaffold’s porosity could also be counted as a potential niche for cells and neovascularization [70–72]. In epidermis layer, a high number of keratinocytes with different cell-junction mechanisms in its sublayers are seen as the main present cells. On the contrary, in the dermis layers, different types of cells (fibroblasts, macrophages, and adipocytes) exist, however, in much fewer amounts (Fig. 1). The potential influences of both continuous and discontinuous interconnectivity gradients on skin cells signaling pathways are suggested here. In addition, doing both biological and engineering studies could help in finding the optimal pore size for different layers of skin. The pore’s shapes and architecture should also be designed based on different existing cell types in each skin layer [73, 74]. Because of the influences of scaffold’s architecture on its stiffness and vice versa, the dual effects of these properties are emphasized.

5.1.1. Scaffolds compositional gradients

The biomaterials composition choice and scaffold design become critical when biomimicking heterogeneous tissues, such as skin with various physicochemical gradients in different layers [20]. Based on the two main layers of skin tissue (epidermal/dermal), several natural, synthetic, and composite scaffolds have been suggested for wound healing. The commonly used natural biomaterials include albumin, fibrin, collagen, gelatin, silk, fibrinogen, glucans, dextran, cellulose, alginic acid, hyaluronic acid (HA), chitosan, heparin, and chondroitin. On the other hand, polyesters, such as polylactic acid (PLA), polylactic acid (PGA), polycaprolactone (PCL), and their copolymers, are the most frequently used synthetic biomaterials for skin regeneration [64]. These bilayer scaffolds have been commonly designed by culturing fibroblast and keratinocyte cells on scaffolds, which have the general requirements suitable for skin regeneration. The cells are commonly cultured on one type of biomaterial for epidermal and the other one or the same for dermal layer [64]. However, these types of bilayer scaffolds might be able to meet the general requirements of skin tissue regeneration; their capability in fitting the existed complex gradients in various sub-layers of this tissue is in doubt. Hence, over the past few years, decellularized scaffolds, which are obtained through the isolation of ECM tissue from surrounding cells, are widely used as natural scaffolds [75–78]. Decellularized scaffolds could already have many of natural tissue features and release growth factors in a gradient controlled manner, making them promising candidates for tissue regeneration [79]. However, their existing challenges, including the ECM differences in donors, as well as donor tissue availability, make their usage difficult. In addition, the regulation of producing, controlling of donor tissues and organs, safety examinations, quality control, and ethics are vital challenges for their commercialization part [80]. Hadler et al. [81] have currently developed a trilayer scaffold based on PCL and gelatin as biodegradable polymers with the aim of stimulating the regeneration of three main layers of skin. For making physical gradients (including compositional, porosity, and mechanical), various fabrication methods, including casting, electrospinning, and lyophilization, were used. Their results indicated that because of the considered gradients in different layers, the designed scaffold could provide a proper microenvironment for concurrent healing of epidermis, dermis, and hypodermis. In a cell co-culture model on both keratinocytes and dermal fibroblasts, the scaffold enhanced their proliferation and differentiation [81].

5.1.2. Stiffness gradient

Researchers have currently designed scaffolds with special stiffness gradients to evaluate their effects on cellular responses. For instance, fibroblasts were placed on a hundred-meter-long styrenated gelatin with a gradient elastic modulus from 10 to 400 kPa. It was detected that cells favorably migrated toward regions with enhancing rate of elastic modulus in 10–80 kPa or 50–300 kPa ranges [82]. In addition, based on the durotaxis or mechanotaxis mechanism, most cell types, including fibroblasts and epithelial cells, migrate from softer to stiffer areas when exposed to a stiffness gradient [21, 82]. Some fibroblasts were located on different soft and stiff polycrylamide scaffolds. Young’s modulus for soft scaffolds was 14 kPa, ~2.7 kPa (on the surface); and 1.8 kPa, ~4.4 kPa (in bulk) and for stiff ones was 30 kPa, ~7.7 kPa (on
Some changes in the cells’ cytoskeletons were observed so that in softer scaffolds, cells had a round morphology in comparison with stiffer ones, where cells had a flat morphology. In addition, Martinez et al. [85] have recently investigated the epidermal cells’ responses to polyelectrolyte multilayers (PEMUs) scaffolds containing uniform and gradients of stiffness by using scales of the fish Poecilia sphenops (Black Molly) and Carassius auratus (Comet Goldfish). Poly(allylamine hydrochloride)-terminated PEMUs (PAH-PEMUs) that were noncrosslinked, UV crosslinked to an unchanging stiffness, or UV-crosslinked with an edge mask or via a neutral density optical gradient filter (for designing nonstop compliance gradients) were

![Diagram](image.png)

**Fig. 2.** The epidermal cells’ responses to polyelectrolyte multilayers (PEMUs) scaffolds containing uniform and gradients of stiffness. (A) The scaffolds cells migrated from Comet Goldfish scales on an uncoated glass (CS) to a Poly(allylamine hydrochloride)-terminated PEMUs (PAH-PEMU) shallow modulus gradient with reducing modulus (arrow shows the gradient’s direction). Comparative positions and migration trajectory plots were plotted for 4 edge cells (1–4) and 6 interior cells (5–10) in the sheet on the uncoated glass coverslip (B) and in the emerging scaffold toward the softer areas (C) with (*) mark as the initial cells’ position and also marking the next positions every 30 s. sheets’ cells migrated toward the stiffest end of the gradient without totally disordering the scaffold. (D & E) Epithelial cell sheets developing from Comet Goldfish scales positioned on the multilayer scaffolds of uniform modulus (D, 120 MPa) and along a 0.65 mm area of a vertical stiffness gradient orientation (E, 1.5 mm; ~90 MPa—~120 MPa, change of ~20 MPa mm⁻¹). The arrow shows extremely extended cells over the area with a vertical modulus gradient. The edge line shows the used mask location for producing the steep gradient. In addition, cells were stained for actin (red) and DNA (blue). Scale bars, 100 μm. Reprinted from Ref. [85] with permission from Elsevier.
Hierarchical nanoporous and interlocked microridge arranged sensors by considering the special gradient elastic modulus of native skin tissue. (A) The gradient stiffness in native skin tissue. The various elastic modulus (E) of skin sublayers with interlocked microridges potentially transfer the exterior stress to the fundamental mechanoreceptors. (B) Hierarchical nanoporous and interlocked microridge arranged sensors. Scanning electron microscopy (SEM) figures of (C) nanoporous and microridge designed PDMS and (D) joined with hierarchically designed P(VDF-TrFE). Reprinted with permission from Ref. [16]. Copyright (2019) American Chemical Society.

employed. Their results indicated that each cell moved linearly, phlhotaxing away from the fish scale (Fig. 2B). In contrast, at the time of orienting scales toward the softer end of gradient, each of cells deviated from the usual linear migration and turned perpendicular to the gradient or moved toward the stiffer gradient site (Fig. 2C). A further robust lamellipodial front was seen in the inferior area of the sheet (Fig. 2A) so that the edge and interior cells' migration curved the migration of sheet's cells from the reducing modulus amounts toward enhancing ones. As can be observed in Fig. 2D and E, the cell sheets migrated toward the stiffer end of the gradient without, totally disordering the scaffold. Overall, the authors revealed that the epidermal layers could potentially accommodate gradients of shear tension, which is important in enhancing tissue regeneration [85].

Ha et al. [16] have currently designed some hierarchical nanoporous and interlocked microridge arranged sensors (Fig. 3B) by considering the special gradient elastic modulus of native skin tissue (Fig. 3A). The sensors were based on poly(dimethylsiloxane)/poly(vinylidenefluoride-cotrifluorethylene) polymers [(PDMS)/P(VDF-TrFE)]. In these sensors, the PDMS layer exhibited open porous networks with a pore size in the range of ~2.2–5 μm (Fig. 3C), while the P(VDF-TrFE) structure was formed by inner pores and nanofiber-like surfaces. In addition, the backsides of sensors were treated with Ag film/Ag nanowires (NWs) electrodes (Fig. 3D). It was demonstrated that sensors were able to potentially improve the compressibility and contact area differences in skin, which was due to the operative transmission of the external stress from stiff to soft layers (inspired by the native skin tissue function). The considered microridges in sensors also provided an operative gap distance difference between the sublayers without using any bulk spacer, which made bio-mimicking the skins sensory receptions in different environments easy [16].

5.1.3. Pore size and porosity gradient

So far, few studies in the skin regeneration field have reported designing a continuous gradient in pore's size and shape, porosity, and interconnectivity in different sublayers [86–88]. For instance, Huang et al. [88] have recently designed some silk-based skin scaffolds with a gradient pore size by combining multistep electrospinning with low temperature (LTE) collection. The gradient pore size was tuned by controlling the silk concentration, electric field, flow rate, needle gauge, and collector temperature during electrospinning at 50% relative humidity (RH). They reported that areas of scaffolds containing smaller pore size (SPL) (average diameter 5.9 ± 1.4 μm) limited the fibroblast proliferation and infiltration. However, the areas with a medium pore size (MPL) (average diameter 11.6 ± 1.4 μm) enhanced cell proliferation. Additionally, the cell's infiltration increased in the areas containing the largest pore size (LPL) (average diameter of 37.2 ± 12.9 μm) (Fig. 4). Overall, they concluded that scaffolds containing different layers of small, medium and large pores could be promising candidates for skin regeneration with gradient pore structures [88]. In another study, a three-layer scaffold containing a pore size gradient was synthesized for dermal regeneration. In this scaffold, the pore size decreased by increasing the scaffold's depth, which resulted in potentially enhancing both granulation tissue formation and wound re-epithelialization [67].

5.2. Chemical signal gradients

Continuous concentration gradients of signaling biomolecules (such as growth factors and adhesive peptides) could also help with enhancing in vivo wound healing and tissue regeneration processes [89–91]. The spatial and temporal biomolecules' release through scaffolds is potentially required for having a successful wound healing in a biomimetic approach. Skin molecular and cellular responses to scaffolds are highly dependent on both absolute and continuous concentration gradients of biomolecules. For instance, during wound healing, fibroblasts, leukocytes, and neutrophils functions are highly governed by the chemotactic factors expressed by macrophages and platelets [92]. Hence, considering the concentration and gradient slope of specific biomolecules are critical at the time of designing skin scaffolds [22]. Chemical gradients are categorized into two groups, including immobilized and soluble factors gradients.

5.2.1. Immobilized gradients

Immobilization could be potentially used for either bio-mimicking an in vivo mechanism or keeping a stable concentration gradient when using a scaffold. Several studies have currently
immobilized various growth factors and adhesive peptides in skin scaffolds in either absolute or slope concentrations to study the resulting molecular and cellular responses [93–97]. The commonly used methods for designing surface-immobilized chemical gradients are the adsorption of specific biomolecules on the scaffolds, covalent binding of biomolecules to the chemical groups of scaffolds’ surface, or through using photo-reactive moieties [21]. For instance, Masters et al. [98] have invented a polymeric wound dressing with a patterned gradient of immobilized growth factors (from low to high concentrations) to improve the cells’ migration during skin regeneration. Their results revealed that skin cells migrated toward the higher amounts of growth factor, and their speed was determined by the gradient’s slope [98]. In addition, Oh et al. [99] have synthesized some cylindrical scaffolds based on PCL/Pluronic F127 by slowly increasing the recombinant bone morphogenetic protein-7 (rhBMP-7), transforming growth factor-β2 (TGF-β2), and VEGF-65 concentrations. They designed the scaffolds by centrifugation of fibril-like PCLs and subsequent use of surface growth factors’ immobilization through heparin-binding with the aim of gradually enhancing the concentrations from top
(BMP-7, 60.89 ± 2.51; TGF-β2, 42.85 ± 2.00; VEGF165, 42.52 ± 3.22 ng/scaffold section) to the bottom layers (BMP-7, 181.07 ± 3.21; TGF-β2, 142.08 ± 2.91; VEGF165, 112.00 ± 4.00 ng/scaffold section) (Fig. 5A). The concentration of growth factors and heparin slowly reduced from the bottom to the top position of scaffold (Fig. 5B-E) [99]. However, for having a successful wound healing, much attention should be paid to the specific skin cells’ responses to continuous biomolecules gradients in biodegradable scaffolds. The following disadvantages of using this strategy has led to the usage of soluble biomolecule gradients. First, the covalent interactions strength depends highly on the protein type, which might restrict the protein selection. Second, immobilized proteins might not be able to start communications with cells. The potential influence of covalent bonding on the molecular and cellular responses is also another drawback of this approach [100].

5.2.2. Gradient in soluble-factors

Soluble biomolecule gradients in skin scaffolds could enhance the biomimicry of wound healing process. First, the molecular diffusion principle has been utilized for designing soluble biomolecule gradients, and thereafter, studying the effects of this type of gradients on cell functions [21,101,102]. However, for more successfully designing scaffolds with continuous concentration gradients of biomolecules, microporous gels with a single or multisource/chamber of biomolecules are currently being used [21,103]. Akar et al. [91] have designed a growth factor gradient in some fibrin-poly (ethylene glycol) (PEG) scaffolds by using a particular leaching method. The platelet-derived growth factor-BB (PDGF-BB) was loaded into poly(lactide-co-glycolide) (PLGA) microspheres, which were positioned distal to the tissue-scaffold interface. The PLGA microspheres could improve the growth factor’s sustained release and its diffusion via the porous scaffolds in a gradient manner. The near-infrared fluorescence imaging confirmed the gradients and diffusion of growth factor within the scaffolds in vivo, which continued for more than 3 weeks and led to improving tissue angiogenesis (Fig. 6) [91].

6. Current strategies for gradient generation in biomaterials

6.1. Traditional strategies

Although solvent casting and particulate leaching techniques are traditionally used for designing homogeneous porous scaffolds, they could be enhanced to generate gradients in interface tissue engineering [104]. Main benefits of using these systems is that they are user-friendly and include the opportunity of synthesizing the various shaped scaffolds with direct control over both porosity and pore size [105], although, the essential use of thermoresistant biomaterials and also the impossibility of designing low-porosity graded scaffolds, broadly restrict using these approaches for synthesizing multigradient scaffolds [106]. Another alternative is using lyophilization for designing scaffolds with multishapes and/or-size gradients, which could be useful for skin regeneration [107]. It has been reported that this strategy could be effective in designing multilayered scaffolds with high control over modeling each layer of tissue [108]. Wang et al. [67] have currently designed a sandwich tri-layered scaffold with various amounts of collagen and hydroxyproline for skin regeneration. Their results demonstrated that the employed gradients could enhance wound healing mechanisms in vivo [67]. Centrifugation is among the simplest approaches for designing continuous gradient scaffolds. In this approach, because the centrifugal force is directly dependent on the distance from the rotation axis, materials in the end part of the container undergo a higher force. However, this method leads to only having a linear gradient, which is not ideal for skin engineering [20,109,110]. Plastic compression is another method,
which could reposition the polymer chains and is applied in both confined and nonconfined setups [20]. In the confined approach, the scaffold is located in a holder containing a permeable membrane, and without creating vortexes makes the incompressible liquids flow to be out of it [111]. However, in the second setup, the scaffold sheet is first rolled and then exposed to the compression through a piston [112]. In order to be in the transverse direction of loading axis, the polymeric fibers then reorder themselves inside the network. The fibers that are located in the superior section of mold experience an instant load, and therefore, a shift toward each other, which leads to the formation of gradually denser polymeric layers with homogeneous orientation. However, due to the upper resistance of denser layers, the polymeric chains in the lower part retains the orientation of its random fibers resulting in achieving a molecular orientation gradient in the scaffold. Freeze-casting/phase separation approach has three basic elements, including a powder, solvent, and polymeric reinforcing agent [113,114]. The mixture of these materials is exposed to a diminishing temperature gradient when freezing and subsequently to low-pressure vacuum drying [115]. Under a temperature gradient, the frozen phase, which is frozen at one part and thermally insulated at the other side, is preserved. Hence, through the temperature gradient profile, nucleated ice crystals grow and form vastly anisotropic ice networks; meanwhile, the front of crystalline growth enforces the powder suspension rearrangement on the contrary side of the ice. This mechanism leads to the generation of a chemical gradient of the powder part [116]. However, this approach is simple, it needs a precise assessment of several parameters, including the shape, intensity, and temperature gradient duration, which makes relying on it for designing continuous gradient scaffolds difficult [20]. Gas foaming is another common approach for designing scaffolds with porosity gradients in which a polymer biomaterial is soaked with gas at high pressure (e.g. 800 psi) to fabricate a sponge-like structure [117]. After quickly releasing the chamber’s pressure, gas bubbles are shaped in the polymer. After completely forming the bubbles, the scaffold converts into a 3D porous structure. The quantity of dissolved gas in the solution regulates the porosity features of fabricated scaffolds. However, by using this method, scaffolds with pore sizes ranging from 100 to 500 μm and porosity, ranging from 60% to 97%, could be fabricated; the scaffolds face weak pore interconnectivity and imperfect pore opening [117–119]. Another simple technique for applying both physical and chemical gradients is selective irradiation. Because of the convenience of regulating the device’s parameters, any shape of gradient, including linear, exponential, sigmoidal, and orthogonal, could be designed with it. This device induces inhomogeneous crosslinking by partial, dynamic, or multiform irradiation of formerly homogeneous photoreactive scaffolds. The scaffold is commonly designed from polyacrylamide/bis-acrylamide solution and then is exposed to UV. The gradient can be induced in the system by changing the time of scaffold exposure to the UV source [20,120]. Additionally, if the polyacrylamide thickness is stable, a stiffness gradient could be
applied to the scaffold through regulating the movement speed of
the system's opaque mask. In spite of its advantages, it is only
applicable to photoreactive materials [20].

6.2. Advanced strategies

6.2.1. Rapid prototyping strategies

Rapid prototyping strategies that are commonly based on the
automatic deposition of layers with the help of computer-based
design models have been currently introduced as alternatives to the
mentioned traditional ones [121,122].

6.2.1.1. Selective laser sintering. Selective laser sintering that ex-
poses a high-power laser source to the fuse particles together is one
of the commonly used rapid prototyping strategies. By using this
method, a multilayered scaffold containing physicochemical gra-
dients with a complicated arrangement could be designed by
substituting the melting and deposition of new polymeric powder.
In addition, highly porous scaffolds with interconnected pores,
which are essential for proper cell responses could be synthesized
by using this method [123]. However, the essential high tempera-
ture for this approach restricts the material choice.

6.2.1.2. Fused deposition modeling. Fused deposition modeling is
another common rapid prototyping method for designing gradient
scaffolds made of thermoplastic biomaterials. With fused deposi-
tion modeling, the gradient is produced in a single system con-
taining overlapped fibers, which are consecutively assembled. In
this approach, physical gradients can be designed in two different
ways. The first is printing 0°–90° oriented fibers with continuous
diameters in layers while varying their relative line spacing. The
second way is altering the fibers' orientation layer-by-layer, which
results in directing the interposed unfilled space for having a
porosity gradient [20]. However, some physical gradients could be
made with this approach; it is not able to create multilayered
continuous gradients. Thus, combining fused deposition modeling
with different extrusion-based processes, such as bioprinting, has
been suggested [20]. A great advantage of extrusion-based pro-
cesses is the possibility of using a wide range of natural and syn-
thetic biomaterials [124–126].

6.2.1.3. Bioprinting. By using extensive computational efforts, bio-
printing is able to combine the anatomical precision with a porosity
gradient [127]. Biochemical gradients are also applied to scaffolds
by using various bioinks. With this approach, various biomaterials
can be individually printed, in both sequential order and correct
position. Hence, scaffolds designed from different compounds with
definite locations can be fabricated [20]. However, because of the
several switch times of required nozzles for alternating the bio-
materials' deposition, the essential time for successfully finishing
the process is strictly extended. The necessity of crosslinking inks
after extrusion also restricts the choice of proper biomaterials. The
concurrent printing of various bioinks results in enhancing the
sequential-extrusion method. In this approach, each bioink reser-
voir is linked to the extruder by vessels, which leads to combining
the bioinks in the precise amount before starting the process [20].
Inkjet printing is a noncontact injection type of bioprinters for
creating spatially structured biomaterials, either by straight cells
locating or influencing cell responses via using biomolecules
[117,128]. Cell behaviors are controlled in a spatially and temporally
controlled manner and ink drops can convert the digital pattern
data onto a scaffold. Because an inkjet printer is able to control the
spatial injection of different biomolecules, cells, and biomaterials, it
can be employed in generating gradient structures. However, its
drawbacks, such as clogging of the print heads and failure in
printing biomaterials gradients within a hydrogel make it difficult
to be used in this field [117]. Zhang et al. [129] have currently
developed a bioink with a composition of collagen, HA, and chito-
san for designing skin scaffolds. In addition, for achieving optimal
mechanical properties and cell responses, both pre-chemical
crosslinking before printing and physical crosslinking after print-
ing were used. Their results indicated that by regulating the con-
centration of tyrosinase, the stiffness and degradation properties
were controlled in the system [129]. A full-thickness skin model
containing pigmentation has been currently designed by using this
approach [130]. Multiple layers of collagen hydrogels comprising
fibroblasts were printed, mimicking the dermal layer and then
crosslinked via neutralization by sodium bicarbonate. Both melano-
cytes and keratinocytes were then printed on the top of the layer
to stimulate skin pigmentation. The bioprinted skin scaffold
simulated the dermal, epidermal, and stratum corneum layers
regeneration. Additionally, at the dermal-epidermal junction, the
melanocytes were observed in the epidermal layer [130]. Overall,
the necessities that a polymer should have to be counted as a
printable one, including the correct viscoelastic properties of bio-
inks and using adequately stable fibers, limits using a wide range of
biomaterials [131–133]. When the biomaterial meets these re-
quirements, an unrestricted diversity of physical gradients can be
achieved with this device. In addition, if different numbers of bio-
inks are used for independently printing various biomaterials,
applying biochemical gradients into scaffolds is also possible [20].

6.2.1.4. Microfluidic lithography. Microfluidic strategies have
currently gained substantial attention for designing scaffolds con-
taining physicochemical gradients with spatial and temporal con-
trol. In this system, the slope and shape of gradients are controlled
by using microchannel systems [134]. Excluding photo-cross-
linking methods, multidimensional scaffolds are designed through
firstly forming concentration gradients of prepolymer solutions
and then stabilizing them by suitable cross-linkers [135,136]. Flow-
based microfluidic platform is one of the strategies that can be
adopted to generate chemical gradients [137,138]. For this purpose,
hydrogels are commonly used, which reduces the produced shear
stress influences by liquids perfusion in the microfluidic platforms
[138]. For designing gradient hydrogels, these tools use stepwise
dilutions at various phases and diffusive mixing [139]. He et al.
[140] have currently suggested an alternative method for designing
gradient hydrogels without the use of gradient producing devices
[140]. They designed a technique to produce centimeter long
poly(ethylene glycol)-diacrylate (PEG-DA) based-hydrogels con-
taining a cell-adhesion ligand, Arg-Gly-Asp-Ser, gradient through
prefilling of a microchannel molded in a PDMS layer with a con-
centration of PEG-DA solution from the outlet of the channel. Then,
they added a droplet of another solution with a higher concentra-
tion of PEG-DA. A passive pump-induced flow caused by flowing
the inlet solution inside the channel and a regressive flow made a
gradient of PEG-DA, which was stabilized by photopolymerization
[140]. Based on the in vitro cell studies, endothelial cells could
attach and spread along the hydrogels in a manner dependent on
the RGDS-gradient profile (The cell attachment reduced by
decreasing the concentration of ligand in the area). Also, the
morphology of the cells changed from round in the lower ligand
concentration regions to well-spread in the greater concentration
areas [140].

6.2.2. Electrosprinning

Electrosprinning is another commonly used approach for
designing multigradient scaffolds. This approach is based on the
ejection of a polymeric jet from the tip of an electrically charged
syringe, followed by its assembly onto a counter electrode, which
leads to designing fibers with sizes ranging from 10 nm to few micrometers [117,141]. Through controlling the electrospinning process, the nanofibers' thickness and orientation can be precisely regulated to fit the tissue structure. The nanofibers can have suitable stiffness, porosity, and a large surface-to-volume ratio, which all lead to improving the fibers' biocompatibility [142]. Additionally, through the surface modification of ejected fibers after electrospinning, a gradient can be applied to the designed fibers [143]. Lin et al. [144] have currently designed a tri-layer chitosan-based skin scaffold by using electrospinning. After freeze-drying the chitosan solution and converting it into porous disks, chitosan and skin scaffold by using electrospinning. After freeze-drying the scaffold had a porous layer (2 mm) to mimic the dermis, a thin film (25–45 μm) as the basement membrane, and a layer of nanofibers (100–200 μm) as epidermis. After only one-week cell culture, a tri-layered skin scaffold mimicking the epidermis, basement membrane, and dermis layers was designed. The nanofiber morphology of chitosan and chitosan-pectin was similar; however, the chitosan-pectin nanofiber was further hydrophilic and swelled than chitosan. In addition, the cells' growth on both nanofibers took one week, with higher amounts in chitosan-pectin than chitosan [144]. Because electrospun scaffolds have small interfibrillar pore sizes, which can impair cellular penetration among fibers, Timmak et al. [86] have recently suggested a new method based on electrospinning, electroblowing, post-processing treatments and lyophilization for designing skin scaffolds with porosity gradients. They mixed submicron-sized soy protein fibers fabricated by electrospinning with larger diameter sacrificial polyethylene oxide fibers made by electroblowing. After that, they introduced post-processing treatments and the lyophilization of scaffolds. The final scaffolds had a pore size gradient ranging from 7.8 ± 2.5 μm to 58.0 ± 23.6 μm. Results obtained from RAW 264.7 macrophages and dermal fibroblasts exhibited that fibroblasts proliferation and spreading enhanced on both the small and large pore sides. However, both cell types penetrated meaningfully deeper into the larger pores than the small pore sides [86].

7. Concluding remarks and future perspective

As discussed above, noteworthy nonhomogeneous features are existing in different layers of skin tissue, which should be addressed when selecting and designing scaffolds for skin tissue engineering applications. The biomimetic and functional tissue regeneration strategies could be combined by incorporating various physicochemical gradients resulting in enhancing immunological responses. In fact, for designing successful skin scaffolds, the possible effects of different physicochemical gradients on each other are critical. As the skin cells in different layers communicate through different complexes, considering stimulating these complexes for starting the communication pathways between cells might be helpful. Because chemical gradients are commonly applied through using controlled drug delivery systems, combining delivery strategies with multigradient generation approaches are also important [20–23]. However, a variety of gradient-generation strategies have been currently introduced for designing gradient scaffolds similar to the native tissues; most of them failed to introduce graded structures with mild changes at the interfaces. Since both continuous and discontinuous types of gradients are present in this tissue, utilizing devices with the ability to support both types of gradients would be more accommodating. Combining different chemical and physical gradients in one scaffold is still a challenging issue, which requires employing more powerful and reproducible gradient generators. Finding an accurate association between the molecular and cellular responses to physicochemical gradients is still a huge challenge and we still suffer from having detailed biological evidence related to the wound healing mechanisms. Since most of the studies have focused on porosity, stiffness, and biomolecule concentration gradients, doing further biological investigations for studying all other possible gradients in more physiologically relevant models, such as hypoxia conditions, is also vital. In addition, a wide range of examinations should be done during applying multigradient strategies to verify the graded structure of skin scaffold and strong adhesion between designed layers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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