Engineered hydrogel-based matrices for skin wound healing

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11.1 Introduction

The management of skin wounds and scars represents a major burden upon world healthcare costs. This has assumed an increasing importance due not only to the aging of the worldwide population, but also to other conditions such as diabetes and obesity [1]. In developed countries, it has been estimated that 1–2% of the population will experience a chronic wound during their lifetime [2]. In the United States alone, chronic wounds affect 6.5 million patients, and it is claimed that an excess of US$25 billion is spent annually on their treatment [3]. Moreover, acute wounds such as serious burns in the United States are associated with 70,000 hospitalizations each year [4]. More than 40% of these patients develop large joint scar contractures, yet the costs involved and the potentially generous medical care do not prevent that many of the affected patients leave hospitals with severe disfigurements with permanent physical, social, and economic effects on them and their families [5].

Skin wound healing comprises a series of complex overlapping phases with an intricate cascade of mechanisms that act together to reestablish the integrity of the tissue, although many times imperfectly so due to excessive scarring [6,7]. Acute wounds are typically associated with complete healing with minimal scarring [8], whereas chronic wounds heal slowly or fail to heal due to a disruption of the wound healing events. These could be repeated injuries or pathophysiological conditions, eg, diabetes, arterial and venous insufficiencies, and frequent infections [9]. Normal wound healing proceeds as a coordinated sequence of the inflammatory, proliferative, and remodeling phases [7]. A temporary fibrin/fibronectin matrix is initially formed for hemostasis. The inflammatory phase is characterized by the recruitment of neutrophils by the action of transforming growth factor (TGF)-β, elastin, and collagen fragments to the wound site for phagocytosis of potential infectious agents [10]. Platelet-derived growth factor is a major chemoattractant for fibroblasts. During the proliferative phase, granulation tissue is formed by the stimulation of capillary formation from existent blood vessels (angiogenesis) by

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vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, and TGF-β produced mainly by the recruited macrophages and fibroblasts. A collagen template made by the fibroblasts gradually replaces the temporary matrix that serves as substrate for the keratinocytes during epithelialization, which is stimulated by epidermal growth factor (EGF) and other factors [11]. The remodeling takes over with the strengthening of the cellular neodermis [11], eventually leading to the formation of an acellular scar, by cross-linking/reorganization of the collagen matrix [12].

Under this context, wound management is adjusted to the wound nature and the condition of the patient. Wound dressings are critical for absorbing excess of wound fluid, maintaining an appropriate wound moisture level, preventing bacterial infection and physical shocks, and providing pain relief. The traditional dressings included bandages, cotton wool, lint, and gauzes, but with the recognition of the importance of a moist wound environment for faster healing [13,14], another class of hydrogel-based wound dressings was developed. Examples of commercially available impregnated hydrogel-based dressings are amorphous hydrogels (eg, AquaSite®, Derma Sciences, Princeton, NJ, USA) or hydrogel sheets (eg, AquaClear®, Hartmann, Heidenheim, Germany). While providing a moist wound healing environment, as well as assisting on autolytic debridement of dry, sloughy, or necrotic wounds, the main expectation of these dressings is to ensure rapid wound closure. Effective control of infection is attained with the use of medicated hydrogel-based dressings mainly containing antimicrobial, antifungal, and antiinflammatory agents such as zinc acetate (AmeriGel®, Amerx Health Care, Clearwater, FL, USA) or antimicrobial silver sulfadiazine (SilvaSorb®, Medline, Mundelein, IL, USA) that combine their release with advanced wound fluid management. A wide range skin substitutes have been developed and clinically used in the attempt to improve healing by playing a more active role in the different stages of the process. Skin substitutes, both acellular (Integra®, Integra LifeSciences, Plainsboro, NJ, USA; Biobrane®, Smith & Nephew, Andover, MA, USA; Alloderm®, LifeCell, Bridgewater, NJ, USA) and cellular, offered as autologous (MySkin®, CellTran, Sheffield, UK; Laserskin™, Fidia Advanced Biopolymers, Abano Terme, Italy) and allogeneic (Graftskin®/Apligraf® and Dermagraft®; Organogenesis, Canton, MA, USA), have been used for the treatment of acute and chronic wounds as well as full-thickness burn injuries [15], although issues such as high cost, poor integration of the substitute, scarring at the wound margins, and lack of differentiated skin appendages are still associated to them [16]. Moreover, in demanding clinical conditions or large wounds, split-thickness autografting remains the gold standard [16]. The dependence on donor site availability [17], the discomfort it causes, and the unsatisfactory outcomes have been instrumental in the search for suitable alternatives. Skin tissue engineering strategies and their elements remain as the strongest and the most promising way to attain full skin regeneration. Major hurdles such as slow preparation time, high production costs, variable engraftment rates, and consequently delayed vascularization are limitations yet to be overcome [16,18].

11.2 Hydrogels attractiveness and achievements in skin wound healing

11.2.1 Hydrogel features

Hydrogels are cross-linked hydrophilic polymeric networks characterized by a high water content and a viscoelastic behavior, facilitating the transport of oxygen, nutrients, and metabolic waste.

In situ–forming hydrogels are especially attractive for wound healing as they acquire the wound site shape by filling the damaged site, thus giving rise to a strong tissue–hydrogel interface [142]. Another benefit relies on the homogeneous incorporation of cells, bioactive molecules, or drugs with precursor molecules before gelation [19]. The in situ–forming hydrogels depend on injectable precursor solutions that form gels by the action of temperature (thermoresponsible), ions (ionic), or ultraviolet (UV) radiation (photocrosslinkable). Moreover, although the ionic concentration of the hydrogels has not been raising major questions, the applied temperature and UV irradiation are potentially harmful affecting cell viability and DNA integrity. Thus, a control of the gelation process, namely, a fast gelation at physiologically acceptable temperatures and in aqueous environment is a demand [20].

Physical, chemical, and biological properties of hydrogels can be tuned by changing their composition, such as the type and amount of polymer, by conjugating polymers with different properties, by modifying the chemical composition of the polymer, or by varying the type of cross-linker and degree of cross-linking [21]. This ability to tune hydrogel properties, eg, size, water content, stiffness, and degradation, enables the preparation of hydrogels according to the wound specificities. Moreover, this allows designing matrices that particularly influence the progression of the healing [22]. For example, through the reduction of the degree of substitution of cross-linking groups, the physical properties of dextran hydrogels were changed [23]. The obtained loose interior architecture promoted the infiltration of endothelial cells, thus improving the neovascularization of burn wounds [22]. That versatility in tuning the properties of the hydrogels has also opened the possibility of recreating some of the features of the native extracellular matrix (ECM), known to have a critical role in many biological processes, including wound healing [24].

11.2.2 Bioactive/medicated hydrogels

Bioactive/medicated hydrogels (Fig. 11.1) seem to be advantageous alternatives designed to target distinct aspects of wound healing [25–27]. Except for the aforementioned hydrogel-based dressings with impregnated antimicrobial, antifungal, and/or antiinflammatory agents, no skin substitutes carry similar or other bioactive molecules. Recent advances in the research of hydrogels, with the biofunctionalization of inert hydrogels, are expected to shift this paradigm. Sophisticated approaches have been thought to provide an improved control of the delivery of incorporated drugs.

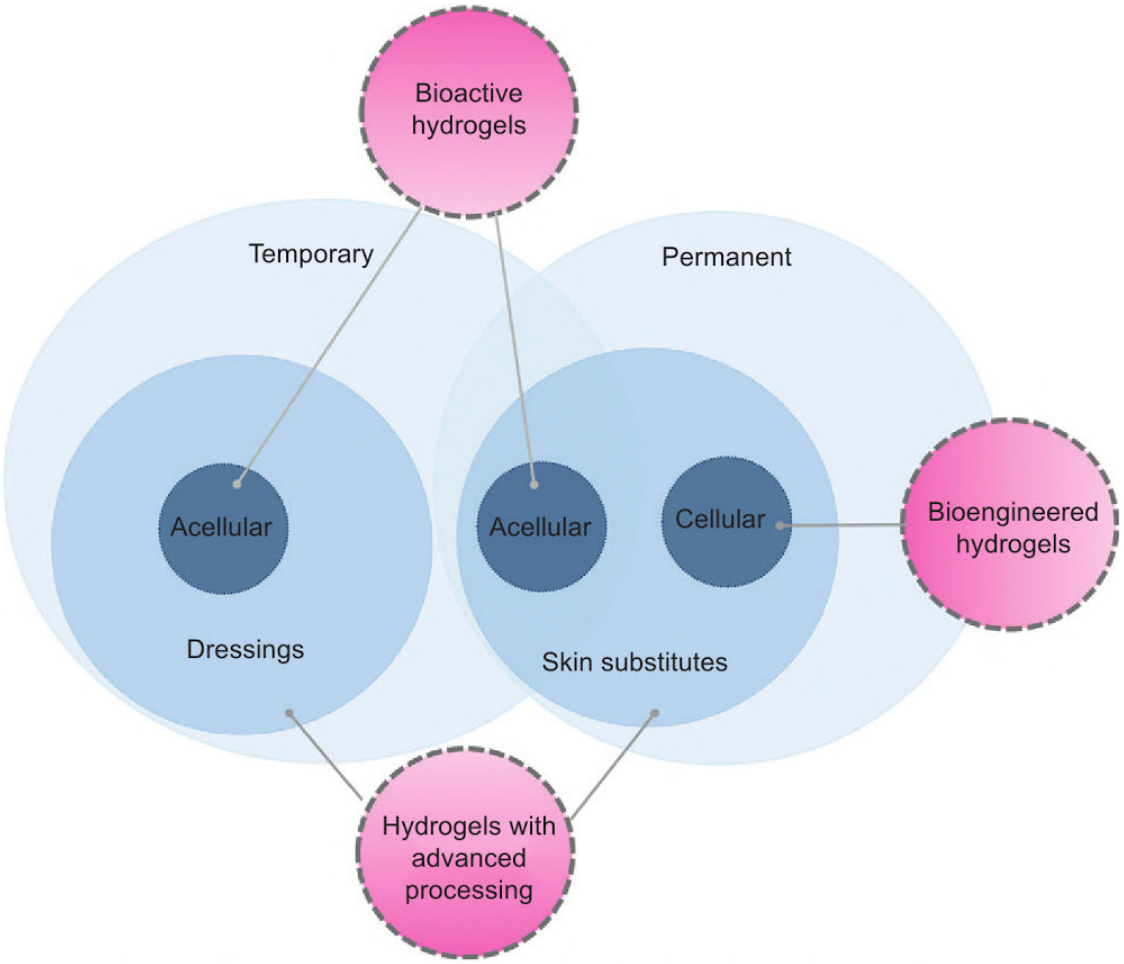

Figure 11.1 Potential application of hydrogels, incorporating bioactive factors (bioactive/medicated hydrogels) or cells (bioengineered hydrogels), and of advanced processing hydrogels differentiated by their enhanced properties resulting from their manufacturing techniques.

Polymeric micelles loaded with curcumin, a potent antioxidant and antiinflammatory agent, were incorporated in a thermosensitive hydrogel, generating a system that could release the drug over an extended period [28]. In fact, the dosage and consequent delivery of drugs incorporated in traditional dressings is problematic, thus strategies that allow a prolonged and controlled release are of outmost relevance. This is also true for other bioactive molecules as their effectiveness depends on the kinetics and the profile of release under the specific conditions of each wound [29]. An in situ cross-linked dextran hydrogel loaded with chitosan microparticles containing EGF and VEGF reduced the size of burn wounds faster than when the growth factors were applied in suspension or freely incorporated in the hydrogel. No statistical analyses were conducted [30].

11.2.3 Bioengineered hydrogels

It is recognized that a suitable three-dimensional (3D) structure capable of supporting an adequate microenvironment contributes to modulate the cellular response by potentiating the dynamics of cellular interactions [31]. Following this rationale, in addition

such as nutrients and oxygen. In hydrogels, cells usually have to degrade their environment to be able to migrate and colonize the entire 3D structure. Consequently, limited molecular diffusivity within the network has been highly associated with poor cell viability. To tackle this issue, different processing techniques (Table 11.1) capable of imposing a rearrangement of the polymeric network and enhancing porosity within hydrogels have been used [44]. Macroporous hydrogels (Table 11.1), created by solvent casting and particulate leaching, involves the use of insoluble salts or other particles (porogens)-saturated aqueous solutions during hydrogel formation that are then dissolved, creating the porosity. Gas foaming (Table 11.1) allows the creation of superporous hydrogels by the addition of a foaming agent, such as sodium bicarbonate or ammonium bicarbonate, to the hydrogels. The nucleation and growth of gas bubbles dispersed throughout the polymer generates a porous microstructure with pore sizes mostly ranging from 100 to 250 μm, which is higher than that in other hydrogels. Other methodologies that take advantage of the freezing thermodynamics were proposed for the generation of xerogels or freeze-dried hydrogels, and cryogels (Table 11.1). The porous sponge-like xerogels, formed by subjecting precursor hydrogels to a freeze-drying process, have the potential to swell and form hydrogels when in contact with aqueous solutions. In opposition, cryogels are hydrogels which are cross-linked at cryogenic temperatures. The formation of solvent ice crystals results in phase separation that then promotes the reaction between the polymer and the cross-linker in solution. After cross-linking, cryogels are thawed and ice crystals (porogens) define the interconnected macroporous network of cryogels, whereas micropores were formed in between the polymer chains.

An effect of wider microarchitecture over cell performance, by means of increasing molecule [45] and oxygen [46] diffusion, promoting differentiation [45,46], migration, and a characteristic organization [47,48], was in fact confirmed in different macroporous hydrogels. Interestingly, an influence on cell proliferation was only observed in different macroporous [49–51] and superporous hydrogels [52–55], and cryogels [56–61] containing cell adhesion moieties to promote initial adhesion.

The arrangement of the polymeric network, creating macroporous structures, directly influences their mechanical stability and elasticity [62]. Together with an enhanced porosity, a facilitated manipulation of hydrogels due to improved mechanical stability, particularly elasticity, would be critical for skin-related approaches. Surprisingly, from the described enhanced processed hydrogels, only gelatin-based cryogels have been suggested to be used for skin wound healing [59,60]. Elastic chitosan/agarose/gelatin cryogels were shown to support the proliferation of fibroblasts [59]. A gelatin cryogel with attached silicone pseudoepidermal layer demonstrated advantages regarding the migration, proliferation, and distribution of fibroblasts, over a 28-day culture period, relative to the clinical gold standard dermal regeneration template Integra®. Furthermore, the formed neoepidermis over the cryogel scaffolds was more mature in comparison to the standard skin substitute. These in vitro results were further proved in a porcine skin wound model, confirming host cellular infiltration, biointegration, and remodeling, and thus supporting the application of developed cryogels as a regeneration template [60].

Table 11.1 **Contrasting properties of standard hydrogels and advanced processing hydrogels**

		Hydrogels	**Macroporous hydrogels**	**Superporous hydrogels**	**Xerogels**	**Cryogels**	**Spongy-like hydrogels**
Physical Properties	Method	Crosslinking	Solvent casting and particulate casting	Gas blowing	Freeze-drying with(out) post crosslinking	Cryogelation	Freeze-drying and re-hydration
	Pore size range (μm)	0.001–50	10–100 100–500 3000–7000	10–100 100–600	20–700	50–300	100–500
	Swelling *(wf-wi/wi)*	5–70	0.2–50	2–140	0.3–30	3–45	13–19
		Slow	Medium-Fast				
		Water diffusion	Water diffusion and capillary rise of water				
	Modulus (kPa)	0.1–150	3–200	3–300	1–100	1–400	10–71
	Shape memory	No	N/A	N/A	Yes [85]	Yes [61,63,86]	Yes [64]
	References	[87–94]	[46–49,95–101]	[102–114]	[85,92, 115–127]	[55,61,63, 128–137]	[64,69,138]
Biological Properties	Cell adhesion	Only with cell-adhesive moieties			Without cell-adhesive moieties		
	Cell migration	Yes [139,140]	N/A	N/A	N/A	Yes [61]	N/A
	Cell proliferation	Yes [139,140]	Yes [47,95,97, 99,101]	Yes [105,108, 110,141]	Yes [120,125, 126]	Yes [60,61, 63,128,142]	Yes [64,69,138]

N/A, not available
Wf, final weight
Wi, initial weight

11.4 Spongy-like hydrogels as advanced matrices for skin wound healing

11.4.1 Spongy-like hydrogels

Spongy-like hydrogels result from hydrogels with specific and enhanced processing [63]. Accordingly, spongy-like hydrogels are formed from gellan gum (GG) hydrogels, after freezing, freeze-drying, and rehydration with a solution containing cells and/or bioactive molecules [63]. Spongy-like hydrogels preserve some hydrogels' characteristic features, such as high water content, yet depicting improved properties such as physical stability, flexibility, handling, viscoelasticity, and recovery capacity [63]. In addition, these materials can be easily available from off-the-shelf dried polymeric networks in varied shapes as they can be stored dry for long periods. At rehydration, spongy-like hydrogels have the capacity to entrap any bioactive molecule, but more importantly they enable adherent cells to bind and proliferate without the use of any peptide cell-adhesive sequence or protein such as collagen or gelatin (Fig. 11.2) [63]. Thus, these cell-compatible GG spongy-like hydrogels capable of supporting the phenotype of different cells for prolonged periods hold potential as soft tissue ECM analogs in tissue engineering strategies.

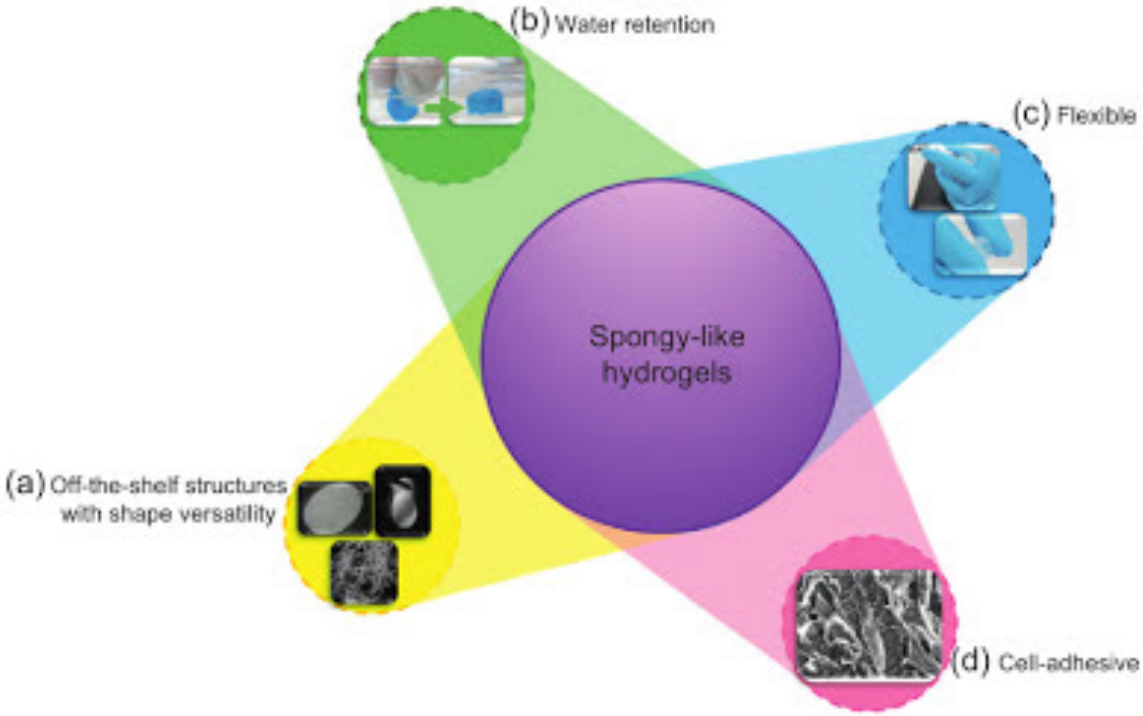

Figure 11.2 Unique characteristics of spongy-like hydrogels. (a) Precursor dried polymeric networks prepared in different shapes can be stored off-the-shelf sterile for months, without losing their specific features. Spongy-like hydrogels are able (b) to absorb large amounts of water within minutes, thus presenting (c) high flexibility, being able to bend without breaking and losing the original shape. (d) When hydrated with a cell suspension, cells become entrapped and are able to maintain their phenotype upon adhesion to the pore walls of the spongy-like hydrogels [63,137].

The first step of spongy-like hydrogels preparation is the formation of GG hydrogels [63]. GG is a particularly attractive polymer for tissue engineering and regenerative medicine applications due to its similarities with the polysaccharides existing in the ECM of native tissues. Although used in many biomedical applications, GG has not yet obtained approval for wound applications. Moreover, GG is susceptible to dual cross-linking mechanisms, ionic and thermoreversible cross-linking (Fig. 11.3), which allows tailoring the hydrogel properties. The involvement of divalent ions, for example, calcium ions at physiological concentration, strengthens the bonding between and the polymeric chains through their carboxylic groups, thus rendering mechanically stronger structures [64]. After gelation, the polyelectrolyte hydrogels are stabilized in a saline solution to achieve osmotic balance, conferring stability to the structure and avoiding the deformation of the network, swelling, or shrinking at the subsequent step of freeze-drying [63]. The second step of spongy-like hydrogels processing involves the freezing and freeze-drying of the precursor hydrogels to attain dried polymeric networks [63]. When the hydrogels are frozen, ice crystals are formed, a process characterized by crystal nucleation and growth (Fig. 11.3). Crystal nucleation starts when solute molecules dispersed in the solvent gather into clusters and solvent solid crystals are formed. Then, crystals grow until the solid–liquid system reaches equilibrium and the crystallization is complete. Crystal formation, specifically their size and shape, are highly dependent on thermodynamic parameters such as temperature, pressure, and solvent concentration [65]. These parameters then determine pore architecture within the polymeric network after freeze-drying, and specifically pore homogeneity, orientation, interconnectivity, and diameter, as previously discussed by us [63]. At this processing stage, the dehydrated hydrogels, termed dried polymeric networks, can be rapidly rehydrated with an aqueous solution containing any bioactive agent [63] or cell, giving rise to spongy-like hydrogels (Fig. 11.3).

Specific parameters along the consecutive stages of spongy-like hydrogels formation, such as polymer concentration and composition, cross-linking solution, stabilization time, freezing temperature, and time, affect their physical and mechanical properties [63]. By varying these specificities in the sequential but integrated processing stages from hydrogels formation into dried polymeric networks, the properties of the spongy-like hydrogels can be tuned.

11.4.2 Spongy-like hydrogels as wound dressings

Spongy-like hydrogels, in contrast to the rigid traditional hydrogels with limited resistance to mechanical stress, can be easily manipulated and transplanted to the patient due to their elastic properties. In fact, the capacity of spongy-like hydrogels to achieve nearly total shape recovery after deformation [63] permits a superior handling for transplantation, not breaking upon the application of a compressive force (Fig. 11.2). Furthermore, their compressive modulus of around 50–100 kPa [63], closely matching the compressive stress module of human skin [66], promotes integration into the wounds.

Long-term stable and available off-the-shelf dried polymeric networks absorb large amounts of water essentially through capillary action within seconds, due to their increased porosity [67] of 100–500 μm, in contrast with the 20 μm of their respective

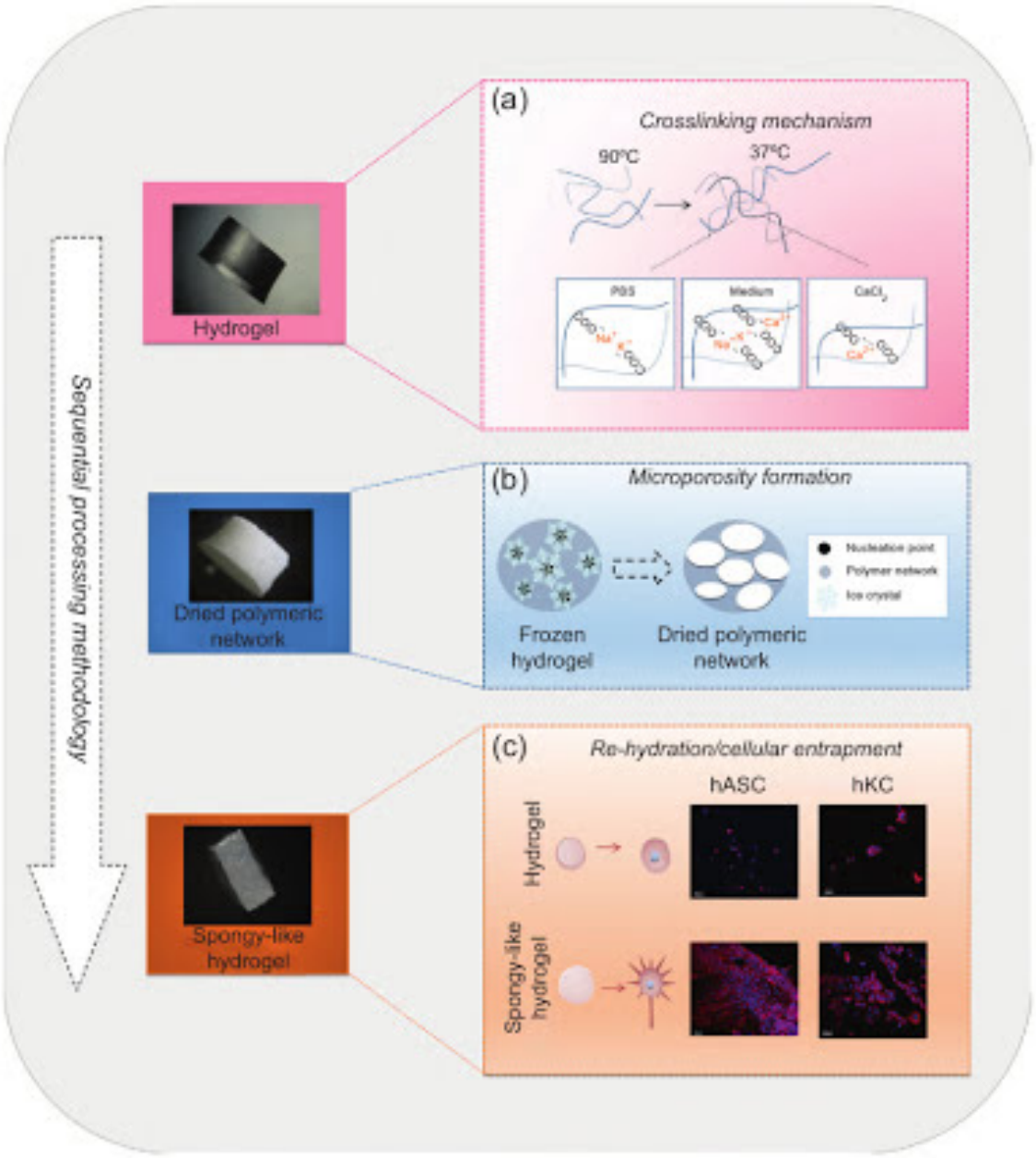

Figure 11.3 Conceptual diagram of the sequential processing methodology to obtain gellan gum–based cell-adhesive spongy-like hydrogels from precursor hydrogels. (a) Gellan gum hydrogels are prepared by thermal (temperature decrease from 90 to 37°C) and ionic (in the presence of phosphate-buffered saline [PBS]) $CaCl_2$ solution, containing mono- and/or divalent ions. (b) Hydrogels are then frozen at −20°C or lower temperatures and freeze-dried to create ice crystals, which determine the porosity of the obtained dried polymeric networks. (c) Rehydration of the dried polymeric networks originate the spongy-like hydrogels that, contrary to the precursor hydrogels, support the adhesion and proliferation of different cell types such as human adipose stem cells (hASCs) and human keratinocytes (hKCs).

precursor (before being freeze-died) hydrogels [63]. Moreover, a rearrangement of the polymeric network and a physical accumulation of polymer around the crystals during hydrogel freezing also contributed to the enlarged pores of spongy-like hydrogels. Thus spongy-like hydrogels maintain the water retention capacity characteristic of hydrogels, as determined in vitro [63]. The absorption capacity of spongy-like hydrogels was also

confirmed in vivo by the volume of exudate observed within the spongy-like hydrogel 3 days after transplantation to full-thickness excisional wounds in mice [68]. Moreover, it further contributed to a progressive degradation of the material which was fully degraded between days 7 and 14. This relatively fast degradation of spongy-like hydrogels is advantageous from the perspective of avoiding a negative interference in the healing progression due to the exchange and/or removal of the spongy-like hydrogels.

The properties of spongy-like hydrogels can be tuned by varying several conditions during processing, including the type of polymers used in combination with GG. HA is one of the major polysaccharides of skin ECM and is highly hygroscopic. A high-molecular-weight HA confers structural support and moisture to the ECM. Spongy-like hydrogels containing HA were shown to promote vascularization [68] and epithelialization of murine full-thickness excisional wounds [68,69]. Low-molecular-weight HA, resulting from biodegradation via hyaluronidase, interacts with different cell receptors and thus indirectly participates in different cell-signaling cascades, including the angiogenic cascades [70–74]. Thus, the capacity of the spongy-like hydrogels containing HA to promote angiogenesis was also confirmed by the 10–15% higher blood flow compared to the control when transplanted into a hypoxic tissue (Fig. 11.4) by using the ischemic hind limb mouse model [75]. The susceptibility of those structures to be degraded into smaller (molecular weight) HA fragments by hyaluronidase was confirmed in vitro by the successively higher amount of low-molecular-weight HA fragments present in the degradation solution up to 28 days, as measured by gel permeation chromatography. Thus the release of low-molecular-weight fragments along the time of implantation into the wound matrix was suggested to play an important role not only in tissue integrity maintenance but also in the neovascularization of the wound.

The high water absorption capacity and improved diffusion and mechanical properties resulting from the increased porosity and polymeric network rearrangement during processing, combined with HA-specific bioactive properties, possibly contributed to the positive influence of GG-based spongy-like hydrogels in skin wound healing. Nonetheless, the possibility of further tailoring spongy-like hydrogels properties, eg, by changing the nature of polymer composition and their ratios, represents an endless number of possibilities that can be explored to meet the purpose of the application and, more importantly, the requirements associated to the nature of the wound.

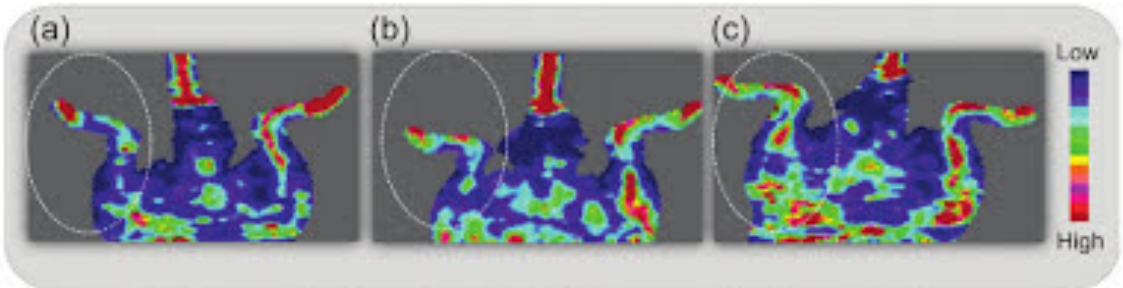

Figure 11.4 Gellan gum-hyaluronic acid (GG-HA) spongy-like hydrogels enhance vascularization 3 weeks after implantation in a hind limb ischemia mouse model. Representative laser Doppler images of the treated limb with the transected vessel area (encircled) and the contralateral intact limb. (a) Control, (b) 1% GG-HA spongy-like hydrogels and (c) 2% GG-HA spongy-like hydrogels.

Likewise, the facilitated incorporation of bioactive molecules within the spongy-like hydrogels, in combination with the intrinsic and tailored physical and mechanical features, will allow a controlled intervention in different stages of the healing.

11.4.3 Spongy-like hydrogels for skin tissue engineering

The lack of cell-adhesive sites in most of the hydrogels, except for those derived from ECM glycoproteins or covalently modified with peptide sequences [76], has limited their use in tissue engineering. Accordingly, GG hydrogels have only showed cell-adhesive properties when combined with gelatin for anchorage-dependent cell delivery [77] or when modified with GRGDS (Gly-Arg-Gly-Asp-Ser) peptide sequences, promoting the adherence and proliferation of neural stem/progenitor cells [78].

In contrast, spongy-like hydrogels are able to entrap or encapsulate and support the adhesion of different adherent cells, which spread within the material, maintaining their typical phenotype, and remaining viable and proliferative (Fig. 11.3). This effect is associated to microstructural rearrangements within spongy-like hydrogels, characterized by pore wall thickening and pore size augmentation, as well as lower water content than precursor hydrogels [63]. These properties significantly affected protein adsorption from the culture media, once the characteristic cell adhesion on spongy-like hydrogels was inhibited in the absence of serum at the rehydration [63]. Nonetheless, the cell-adhesive character of spongy-like hydrogels also depends on the cell type. Human epidermal keratinocytes (hKCs) and endothelial cells cultures are highly dependent on adhesive substrate coatings. Interestingly, spongy-like hydrogels similarly supported hKC and human ASCs (hASCs) (Fig. 11.3) and osteoblastic-like cells adhesion but not endothelial cells. In normal skin, keratinocytes are bound by cell junctions promoted by the connection of the $\alpha_6\beta_4$ integrin to laminin-332 at cell–substrate contacts. During epithelialization, the migration of keratinocytes is influenced by cell attachment to a provisional matrix composed of fibrin, fibronectin, vitronectin, and laminin-332, as well as to dermal collagen. Likewise, in vivo vasculogenesis and angiogenesis are dynamic biological processes in which endothelial cells binding to fibronectin determine blood vessel formation. A preincubation of the spongy-like hydrogels with fibronectin promoted the adhesion of endothelial cells within spongy-like hydrogels [63]. Thus, although further studies are necessary to elucidate the mechanisms involved in this selective cell adhesion, the results so far [63] suggest that fibronectin is not one of the proteins that is adsorbed from serum into spongy-like hydrogels.

Along with the favorable cell-adhesive properties, the inclusion of HA adds new features to the spongy-like hydrogels. As an additional resemblance with skin ECM, we also expected to maximize skin cells potential. Thus, human skin cell fractions, dermal and epidermal fractions freshly isolated, were entrapped within the spongy-like hydrogel, aiming at targeting epithelialization and hypothesizing that the recreated environment would enable cell self-organization after transplantation into full-thickness wounds created in immunocompromized mice [69]. Moreover, hASCs and human adipose microvascular endothelial cells (hAMECs), both isolated from human adipose tissue, were entrapped in spongy-like hydrogels with the goal of assessing a synergistic contribution of HA fragments and stem and endothelial cell secretomes toward early neotissue vascularization [79].

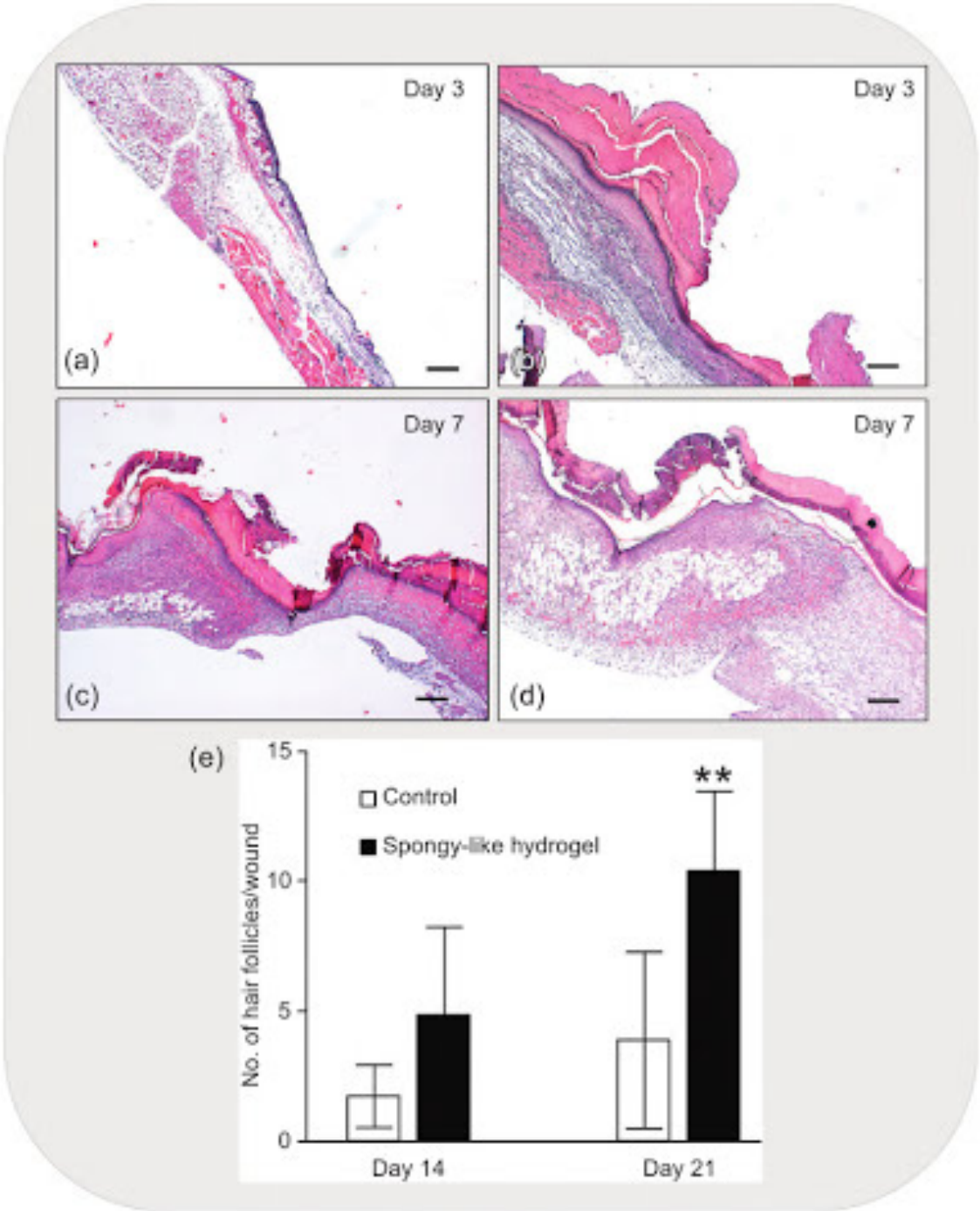

Figure 11.5 Effect of the gellan gum-hyaluronic acid (GG-HA) spongy-like hydrogel with human dermal (1.25×10^6) and human epidermal (0.25×10^6) cell fractions on repair (a-d) and hair follicle formation (e) in murine full-thickness skin wounds. The construct was cultured for 2 days before being applied to the 1.2 cm circular wounds on day 0. The construct was covered with Hydrofilm (Hartmann). Wounds in control animals received Hydrofilm alone. The male mice were given prednisolone (20 mg/kg) postoperative days 0, 7 and 14 to delay wound healing. Four animals were used per group and time-point. (a, c) Control wounds and (b, d) wounds treated with the experimental GG-HA construct postoperative days 3 (a, b) and 7 (c, d). Experimental wounds showed earlier epithelialization than control wounds. Hematoxylin-eosin stain. Scale = 50 µm (a-d). (e) Formation of hair follicles in the wounds postoperative days 14 and 21 (mean ± SD). Significantly ($^{**}p < 0.01$) more hair follicles were observed by light microscopy in the experimental compared with the control wounds on postoperative day 21 [69].

It was also our major concern in both approaches to propose a construct that combined an off-the-shelf matrix with readily available short-term cultured cells. Unlike 3D dermal-epidermal substitutes that need fastidious and complex cell isolation procedures, the spongy-like hydrogels-based approaches avoid extensive in vitro cell culture. GG-HA spongy-like hydrogels acted as a 3D support for the entrapment of the human cells, but also allowed their delivery at the wound site and integration into the neotissue forming underneath, suggesting their contribution to wound closure, with a sustained epithelialization. Moreover, a significantly increased number of hair follicles in the wound tissue was detected at later time points (Fig. 11.5). Our findings suggest a synergistic effect of the matrix and the entrapped cells in different ways, confirming that spongy-like hydrogels provided the adequate environment for cells to respond to the host signals and reach the wound bed in a timely manner. When human dermal/epidermal cells were entrapped within GG-HA spongy-like hydrogel directly after isolation and applied to full-thickness wounds in immunocompromized mice [69], a significant synergistic effect of the matrix and human cells on epithelialization and angiogenesis was demonstrated, mainly at early time points. The GG-HA spongy-like hydrogel acted as a suitable supporting matrix for the transplanted cells during the early time points, allowing them to contribute to the early epithelialization and neovascularization (Fig. 11.5). When spongy-like hydrogels were combined with stem cells and endothelial cells derived from human adipose tissue, angiogenic units capable of promoting neovascularization were generated. A cumulative effect of the GG-HA spongy-like hydrogel matrix and the hAMECs incorporated in the heterotypic constructs (hASCs and hAMECs) was detected by an improved neovascularization of murine full-thickness skin wounds. Moreover, the hAMECs were integrated in the new vasculature, demonstrating an active role of the transplanted cells in the healing process (Fig. 11.6). However, we were unable to confirm our hypothesis; the isolated

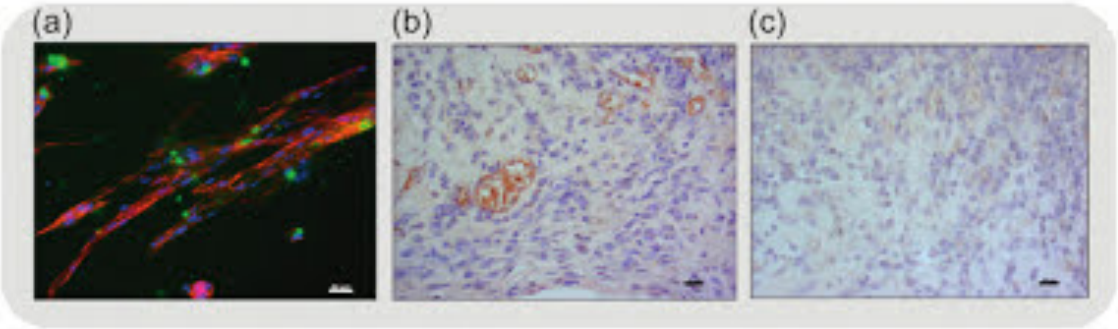

Figure 11.6 Engraftment of human adipose microvascular endothelial cells (hAMECs) in 2% gellan gum-hyaluronic acid (GG-HA) spongy-like hydrogel with or without human adipose stem cells (hASCs) applied to murine full-thickness excisional wounds day 0. The male mice were given prednisolone (20 mg/kg) days 0, 7, and 14 to delay wound healing. (a) Organization of the construct with hAMECs (5×10^5) cocultured with hASCs (1×10^5) for 2 days before being applied to the 1.2-cm circular wounds. The interaction between the hAMECs and hASCs is clearly shown by the von Willebrand–positive hAMECs (*green*) and the F-actin fiber–positive hASCs (*red*). (b, c) Integration of the hAMECs, demonstrated by the positive CD31 immunostaining (*brown*), in the neovasculature of the wound tissue postoperative day 21 in the presence (b) or absence (c) of hASCs in the construct [68]. Scale = 50 μm (a), 20 μm (b, c).

cellular fractions of early differentiation-stage keratinocytes, fibroblasts, and endothelial cells, together with the proposed matrix, were not capable to self-organize in a 3D epidermal/ dermal structure after transplantation. This suggests that the spongy-like hydrogel system did not succeed in prolonging residence time of cells and in providing the necessary cues for the migration and rearrangement of the transplanted cells to form the neotissue.

These examples highlight the potential of spongy-like hydrogel cell-adhesive structures to be used in the context of skin tissue engineering. The possibility of combining a wide range of cellular components, as homo- or heterotypic systems, and potentially controlling their arrangement through the matrix microarchitecture and thereby modulating cell–cell and cell–matrix interactions, is clearly favored in spongy-like hydrogels in relation to standard hydrogels.

11.5 Future trends

The efficient treatment of skin wounds toward a fully regenerated skin is still under development as the distinct wound dressings and the clinically available skin substitutes are not able to fulfill the requirements of a successful healing.

Due to the intrinsic properties of hydrogels and the possibility of tuning their physical, chemical, and biological properties to enable their preparation according to the wound specificities, the relevance of hydrogels as wound dressings and matrices of bioengineered skin substitutes is unquestionable.

A clear intention to drive hydrogels from dressings, whose main goals are to prevent the occurrence of infection and sustain wound moisture, into platforms for skin regeneration have been demonstrated (Fig. 11.1). However, the optimal properties of the hydrogels and their combination with other molecules and cells are yet to be defined. Moreover, considering the variation among the types of wounds and patients, it is important to highlight that no single combination is suitable for the regeneration of all wounds, and for efficient targeting of all phases of the wound healing process.

Hydrogel-based dressings are available as medicated structures, incorporating drugs mainly to prevent wound infections. However, more sophisticated strategies aimed at providing an improved control of the delivery of incorporated drugs are still required, and efforts to include other bioactive molecules relevant for the different healing stages need to be initiated. Despite the improved properties of the enhanced processing hydrogels, it seems that the dependence of the presence of a cell-adhesive site to promote initial cell adhesion is restraining their application, particularly in skin wound healing. In fact, whether the capacity of cells encapsulated within hydrogels to trigger local responses toward a well-coordinated wound healing response has been maximized is uncertain, as most of the hydrogels lack cell-adhesive properties [76]. Therefore, most of the nonmodified hydrogels act as cellular delivery systems in which cell–cell interactions, but more importantly cell–matrix interactions, are limited.

Spongy-like hydrogels may be promising alternatives to hydrogels, retaining their attractive features but adding improved physical properties, and most importantly a cell-adhesive character. These structures also permit to overcome issues such as

adverse cross-linking conditions that might compromise the encapsulated cells once spongy-like hydrogels result from a prompt rehydration of a dried polymeric network. Thus a rapid combination with cells or bioactive molecules/drugs, of major importance in the clinic, is possible with spongy-like hydrogels, but not conceivable with standard hydrogels.

The potential of spongy-like hydrogels for skin repair and regeneration has been explored by taking advantage of different cellular players, skin cell lineages and stem cells, with the particular aim of targeting epithelialization and neovascularization in full-thickness skin wounds. The possibility of changing the polymer composition in the spongy-like hydrogels, thus further tailoring their properties, opens the possibility to meet the specific requirements of the healing of wounds with different natures (Fig. 11.1). The facilitated incorporation of bioactive molecules within the spongy-like hydrogels allows envisioning a controlled intervention in different stages of healing or the targeting of more complex and specific skin disorders. For example, growth factors such as EGF [25] and FGF-1 [80], keratinocytes-specific protein stratifin [81], cytokine stromal cell-derived factor-1 [82], and neuropeptides [83] that have been topically applied for the treatment of diabetic foot ulcerations can be more efficiently delivered into the wound if entrapped within spongy-like hydrogels.

Although an area with the longest history of products in the market, skin tissue engineering is still searching for satisfactory solutions for patients needing urgent wound care, but also toward providing definitive solutions for a regenerated high-quality skin. Strategies with integrated elements such as cells, growth factors, and an adequate matrix, such is the case of spongy-like hydrogels in which both cell–cell and cell–matrix interactions are favored, remain of major importance to provide an ECM-like microenvironment for a more effective and integrated responses.

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