

The contribution of immunohistochemistry to the development of hydrogels for skin repair and regeneration

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ABSTRACT

Hydrogels based on various polymeric materials have been successfully developed in recent years for a variety of skin applications. Several studies have shown that hydrogels with regenerative, antibacterial, and anti-inflammatory properties can provide faster and better healing outcomes, particularly in chronic diseases where the normal physiological healing process is significantly hampered. Various experimental tests are typically performed to assess these materials' ability to promote angiogenesis, re-epithelialization, and the production and maturation of new extracellular matrix. Immunohistochemistry is important in this context because it allows for the visualization of *in situ* target tissue factors involved in the various stages of wound healing using antibodies labelled with specific markers detectable with different microscopy techniques. This review provides an overview of the various immunohistochemical techniques that have been used in recent years to investigate the efficacy of various types of hydrogels in assisting skin healing processes. The large number of scientific articles published demonstrates immunohistochemistry's significant contribution to the development of engineered biomaterials suitable for treating skin injuries.

Key words: Immunohistochemistry; hydrogels; healing biomarkers; skin repair; skin regeneration.

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Introduction

Skin is the outer organ of vertebrates and, covering the whole body, represents the first line of contact with the environment. From the functional point of view, skin controls body temperature, detects sensation from external stimuli and acts as a protective barrier against physical, chemical and biological noxious agents, thus ensuring the maintenance of body homeostasis. Due to its protecting role, skin is vulnerable and its integrity can be easily compromised by several external factors causing injury, burns, microbial invasion, inflammation, premature aging and even cancer.¹⁻³

In healthy individuals the process of cutaneous repair is very efficient. However, in chronic diseases such as diabetes, arteriosclerosis and vasculitis, the skin regenerative capability is significantly impaired; moreover, serious traumatic injuries (*e.g.*, burns) hardly heal. In these cases, innovative solutions and therapeutic approaches are required to promote faster healing and restore skin functions. To this aim, different strategies have been proposed such as cytokine or stem cell therapy, autograft, allograft, xenotransplantation, and engineered skin substitutes.^{4,5}

Among these approaches, skin substitutes are especially effective due to their easy availability, low risk of infection, reduction of scarring and reduced need for surgical procedures.⁶ Many materials in different forms have been investigated as skin dressing for tissue regeneration, such as films, patches, sponges, sheets, foams, nanofibres, hydrogels.⁷ Among them, hydrogels represent a very promising tools in the field of tissue engineering thanks to their unique three-dimensional (3D) structure, good permeability, low toxicity, easy preparation and application, suitability in absorbing wound exudates, and ability to provide a moist environment able to stimulate healing and patient compliance. Hydrogels have also been used for the 3D reconstruction *in vitro* of human skin for experimental purposes.⁸⁻¹⁰

Considering the increasing interest on hydrogels designed for skin repair and regeneration, this paper aims to present an overview of the most recent progresses in the development of high-performance hydrogels, highlighting the great contribution of immunohistochemistry (IHC) to the evaluation of the therapeutic potential of these innovative medical devices.

Application of hydrogels to skin wound healing and diseases

Through an accurate preparation and rational design, it is possible to obtain hydrogels endowed with specific functionalities useful for various skin applications. Hydrogels can be broadly classified according to either the material used (producing natural, synthetic or hybrid hydrogels) or the synthesis methods.

One of the most common applications is to support the regenerative process in different skin injuries such as chronic wounds, deep ulcers or burns, and in general skin loss. In the context of damaged skin, numerous biomimetic gels have been developed and modified to match the timescale for the distinct stages of acute wound healing (Table 1). These materials represent a promising dressing for skin treatment thank to their ability to act on a single or on various stages of the healing process. Since hydrogels act as temporary skin substitutes, they must be sterile, biocompatible, biodegradable/removable, non immunogenic, impermeable to exogenous bacteria, able to firmly cover the open wound thus guaranteeing a protective microenvironment.¹¹ All these properties can be achieved by designing gels with specific physico-chemical characteristics or by adding sensitive agents. It is also possible to

develop hydrogels able to respond to specific external physical stimuli.¹¹ For example, Zheng *et al.* developed an injectable pre-gel solution whose elastic modulus drastically outweighs the viscous modulus at 32°C, thus leading to phase transition from liquid to gel and providing a suitable cell survival environment.¹² Moreover, hydrogels can be employed on the injured skin as space filling agents, 3D framework for cells, or delivery support for bioactive molecules.¹³ For example, some injectable hydrogels have shown a rapid adaptability to the irregularly shaped defects of the damaged skin, ensuring a complete cover with minimally invasive injection procedures.^{14,15} Other hydrogels were tailored to deliver bioactive molecules able to accelerate the healing process, angiogenesis, re-epithelialization as well as the production of extracellular matrix. The application of therapeutic substances incorporated into the hydrogel structure protects these compounds against the action of the local enzymes while ensuring their long-term efficacy by controlling their sustained release.^{12,13} Another possibility is the use of hydrogels containing nanoparticles¹⁶⁻¹⁸ or microspheres¹⁹ as drug-delivery systems.

Also cell-loaded hydrogels may provide a therapeutic support in all stages of wound healing. For example, hydrogels enriched with human dermal fibroblasts and epidermal keratinocytes have been shown to accelerate the wound healing thanks to the ability of fibroblasts to secrete cytokines, growth factors and extracellular matrix components promoting the proliferation of the epidermal cells.^{20,21} Other studies proved the potential of undifferentiated stem cells to enhance the immunoregulation, release of the regenerative secretome, angiogenesis and cell recruitment in wound healing.²²

A further type of healing-promoting procedure is based on hydrogels enriched with platelet-rich plasma (PRP).²³ Indeed, PRP plays an essential role during the inflammatory phase by providing several cytokines involved in the regular healing process.²⁴

Finally, the polymers forming hydrogels can be also able to influence the different healing stages of injured skin by acting on cell proliferation, migration, maturation and differentiation, angiogenesis, cell signalling and inflammation.^{5,15,25-27}

In chronic wounds caused by diseases such as diabetes, malignant tumours, infections, and vasculopathy, the physiological cascade of events occurring during the healing process is significantly compromised causing persistent infections, inflammatory response, loss of angiogenic potential, reduced extracellular matrix, and degradation of growth factors. To overcome these drawbacks, Kamar and collaborators developed a hydrogel loaded with curcumin nanoparticles that was able to improve the healing process in diabetic rats by promoting re-epithelialization, dermo-epidermal junction formation, dermis reorganization with significant increased of aquaporin-3 (AQP3), vascular endothelial growth factor (VEGF) expression and collagen deposition.¹⁸ Also hydrocolloid dressing proved to promote angiogenesis, re-epithelialization and extracellular matrix remodelling in diabetic mice with full-thickness defects.²⁸

Moreover, in many chronic wounds the inflammatory response is impaired, leading to the development of important infections: here, the inability of immune cells to eliminate infection represents the main cause of persistent inflammation. To overcome these issues, several hydrogels made of or combined with antiseptic/antibacterial materials, and gels with immunoregulatory activity have been successfully formulated for wound healing and dermatological purposes (Tables 2 and 3).^{26,29-32} For example, Lacatusu *et al.* developed lipid nanostructured carriers-based hydrogels loaded with natural bioactive compounds able to improve the local antioxidant and anti-inflammatory activities in inflamed skin.¹⁷ Hydrogels also proved to be beneficial to burn

wounds, with high-quality and efficient scar inhibition thanks to their water reserving, moist environment and antibacterial properties.^{19,20} Finally, hydrogels also represent a spearhead in the cos-

metics industry. For example, polysaccharide-rich hydrogels proved to successfully reduce the UV-induced photodamage in the skin of mice.³³

Table 1. Hydrogels recently developed for regenerative purpose.

Regenerative hydrogels				
Hydrogels	Main properties	Applications	Animal models	References
SIKVAV (Ser-Ile-Lys-Val-Ala-Val) peptide-modified chitosan hydrogel Type: Composed/functionalized hydrogel	Promotes and regulates cell proliferation, migration, maturation, differentiation and cell signaling. Antiallergenic, antimicrobial and angiogenic properties.	Wound healing	Mouse full-thickness skin defect model	25
Apelin-13-loaded chitosan (CH)/β-sodium glycerophosphate (β-GP) hydrogel Type: Hydrogel as controlled delivery system	Promotes angiogenesis, anti-inflammatory and antioxidant properties.	Wound healing	Rat skin flap model	12
Polysaccharide-rich hydrogel formulation extracted from <i>Lycium barbarum</i> fruits (LBP) Type: Composed/functionalized hydrogel	Protects against oxygen-mediated damage.	UV photodamage	Mouse skin	33
Acellular blood vessel matrix (BVM) hydrogel Type: Decellularized hydrogel	Free of the major histocompatibility complex antigens, rich of the natural extracellular components, biocompatible, bioactive with pro-angiogenic properties.	Skin regeneration; potentially employed for ischemia-related diseases	Rat skin flap model	14
Hydrogel loaded with curcumin nanoparticle (Cur-NP/HG) Type: Hydrogel as controlled delivery system	Anti-inflammatory, antioxidant, antimicrobial properties and wound healing activities.	Diabetic wound healing	Full thickness excisional skin wound in diabetic rats	18
Hydrogel loaded with Cu (II) Schiff base 8-hydroxy quinoline complex (CuSQ) solid lipid nanoparticles Type: Hydrogel as controlled delivery system	Antimicrobial, anti-inflammatory, antioxidant, analgesic and antibacterial properties.	Wound healing	Excision wound in rats skin	16
DuoACTIVE®ET (hydrogel; ConvaTec Japan) Type: Hydrocolloid	Promotes angiogenesis, increases macrophage polarization to the M2 phenotype, promotes migration of epidermal cells, accelerates healing while preventing the adhesion to wounds and scab formation.	Diabetic wound healing	Full-thickness skin defects in diabetic mice	28
Metformin Hydrochloride (MET-HCL) hydrogel Type: Composed/functionalized hydrogel	Ability to regulate some signaling pathways which affect macrophage polarization.	Wound healing	Wound in rats; Clinical study on patients having lower limbs traumatic wounds and cutaneous ulcers	5
Hydrogel including Bacopa Procumbens Type: Hydrogel as controlled delivery system	Regulation of cell proliferation, adhesion, differentiation, migration, and reduction of scratch area.	Wound healing	Excision wound in rats	37
Multi-functional carboxymethyl chitosan/hyaluronic acid-dopamine (CMC/HA-DA) hydrogel, crosslinked by horseradish peroxidase and hydrogen peroxide Type: Composed/functionalized hydrogel	Hydrophilic, adhesive, hemostatic, injectable, antioxidant and biodegradable.	Wound healing	Skin injury in mice	15
Hybrid hydrogel composed of chitin and β-glucan Type: Composed/functionalized hydrogel	Enhances the immune responses, modulates the inflammation processes by stimulating the production of neutrophils and macrophages and inducing the recruitment of leukocytes.	Wound healing and scarring	Skin injury in mice	27
Acellular ovine tendon collagen skin patch made of collagen hydrogel with dermal fibroblast conditioned medium (Col-DFCM); Ovine collagen sponge scaffold with freshly harvested skin cells (OTC); Platelet-rich-plasma (PRP) gel with freshly harvested skin cells Type: Composed/functionalized hydrogel	Col-DFCM: Cell migration and immunogenic properties. OTC: Low immunogenic, biodegradable and versatile. PRP: Strong ability to regulate healing procedure.	Wound healing	Skin injury in sheep	23

Immunohistochemistry for hydrogel development

To provide information on the effects of hydrogels designed for skin applications, different techniques are commonly used. Among these, IHC is the method of choice for the localization and semi-quantification of protein expression in tissue and cell components of the skin.

In injured skin, wound healing is the common physiological process governed by a sequence of phases corresponding to inflammation, proliferation and remodelling. In normal healing, all these stages are characterized by the activities of multiple cell types and messenger factors able to restore the homeostasis and proper tissue/organ function.^{34,35} These mechanisms are impaired in traumatic or chronic injuries and, in this case, hydrogels may be a helpful tool to restore the normal wound healing process. The immunohistochemical detection of wound healing biomarkers is essential to assess the suitability of hydrogels for skin repair and regeneration. Most studies have been focused on factors involved in promoting fibroblast proliferation, extracellular matrix remodelling and angiogenesis, including cell growth factors among which transforming growth factor- β (TGF- β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial cell growth factor (VEGF), hepatocyte growth factor (HGF), and interleukins such as IL-6 and IL-22 (Figure 1). All the studies considered in this review applied IHC at light microscopy, sometimes using fluorescent markers^{21,23} but more frequently using enzyme-based revealing systems,^{12,14-16,18,25,27,28,31,33} probably because of the intrinsic autofluorescence of the skin that prevents the visualization or leads to underestimation of the signal arising

from the fluorophore bound to the antibody.³⁶

There are various examples of application of IHC to detect markers of the wound healing phases. The expression of tumour necrosis factor (TNF- α), a pro-inflammatory cytokine released by platelets during the inflammatory stage, was investigated by Abou El-ezz *et al.* in formalin-fixed paraffin-embedded (FFPE) rat skin sections by using secondary horseradish-peroxidase (HRP)-labelled antibodies and diaminobenzidine tetrahydrochloride (DAB) to develop the colour. The immunopositivity for TNF- α was digitally quantified as the percentage of labelled area in the wounds. IHC staining revealed that hydrogels loaded with the Cu (II) Schiff base 8-hydroxy quinoline complex were able to decrease inflammation; moreover, using the same detection and quantification methods, the authors found an increase of VEGF, that is known to promote angiogenesis and improve vascularization.¹⁶ Similarly, IHC was used in other investigations to evaluate the levels of VEGF in healthy and diabetic rat models of wound treated with Apelin-13-loaded chitosan (CH)/ β -sodiumglycerophosphate (β -GP) gel or hydrogel loaded with curcumin nanoparticles (Cur-NP/HG).^{12,18} In particular, the rat flap zones were subjected to IHC staining using streptavidin-peroxidase/DAB method and the images, collected with an inverted microscope, were used to measure the integral absorbance of the VEGF level.¹² Similarly, Kamar *et al.* incubated FFPE rat skin sections with an anti-VEGF primary antibody and biotinylated secondary antibody, and the area of VEGF-positive staining was measured.¹⁸ IHC staining of CD34 and AQP3 was performed by the same authors with similar samples and techniques to evaluate the cell differentiation and maturation occurring during the proliferative phase of the healing pro-

Table 2. Hydrogels recently developed for antibacterial purpose.

Anti-bacterial hydrogels				
Hydrogels	Main properties	Applications	Animal models	References
Bacterial cellulose/acrylic acid (BC/AA) hydrogel leaded with human epidermal keratinocytes (HEKs) and dermal fibroblasts (HDFs) Type: Hydrogel used as cell carrier	High purity, high water holding capacity, high hydrophilicity, high crystallinity, high porosity and porous 3D structure able to supports cell proliferation and penetration.	Burn wound healing	Burn wound in mice	20
Bacterial cellulose (BC)/acrylic acid (AA) hydrogel carrying human epidermal keratinocytes (EK) and dermal fibroblasts (DF) Type: Hydrogel used as cell carrier	Hydrophilic, flexible, non-toxic, porous structure, good mechanical strength, ability to maintain appropriate moisture balance and prevent bacterial infections.	Wound healing	Skin wound in mice	21
Three-dimensional reticulated antimicrobial peptide (AMP) NZ2114 hydrogels developed based on hydroxypropyl cellulose (HPC) and sodium alginate (SA) Type: Composed/functionalized hydrogel	Antibacterial activity.	Wound healing	Mice full-thickness skin defect model	31
Dextran-hyaluronic acid (Dex-HA) hydrogel enriched with sanguinarine (SA) incorporate in the gelatin microsphere (GMs) Type: Hydrogel as controlled delivery system	Antimicrobial, anti-inflammatory, and antibacterial activities.	Burn wound infection	Rats full-thickness burn infection model	19

cess.^{12,18} In addition, the level of CD34 expression was measured by Zheng *et al.* through the integral absorbance as previously reported for the VEGF.¹²

Epidermal maturation has also been investigated in sections of mouse and sheep skin, by evaluating the immunolabelling for cytokeratin 14 (CK-14, a marker of the proliferating basal keratinocytes), and for involucrin and CK10 (two markers linked to the final maturation stage of the epidermal cells): fluorescent secondary antibodies were here used, and images taken by confocal fluorescence microscopy.^{21,23} The re-epithelialization process was also studied by Kang *et al.* on mouse skin through the immunodetection of CK14 and CK15 using an HRP-conjugated secondary antibody colour-developed with DAB.²⁷

Another essential process that occurs during the proliferative phase is the formation of new blood vessels: this process can be easily monitored by IHC, by detecting angiogenic markers such as FGF, TGF- β , the hypoxia-inducible factor (HIF), VEGF and

CD31: a semi-quantitative assessment of the angiogenic process was obtained by measuring the integral absorbance and mean optical density of the immunostaining and by counting the new blood vessels per square millimetre area as an estimate of the microvessel formation.^{14,16,25,31}

Biomarkers related to the remodelling phase of wound healing have been also widely studied through IHC to set up hydrogels for skin applications. This phase is mainly characterized by wound contraction and collagen deposition. In the granulation tissue, fibroblasts are activated to become myofibroblasts, which possess contractile ability mediated by stress fibres made of alpha-smooth muscle actin (α -SMA). Thus α -SMA is a suitable marker used in several works to evaluate hydrogel ability to enhance cutaneous wound healing. Many authors applied secondary antibodies conjugated with Alexa Fluor probes, or with streptavidin-biotin complex followed by chromogenic detection with DAB, in FFPE mouse skin samples.^{15,21,25}

Table 3. Hydrogels recently developed for anti-inflammatory purpose.

Anti-inflammatory hydrogels				
Hydrogels	Main properties	Applications	Animal models	References
Thermoreversible 15d-PGJ2-poloxamer (PL) 407 hydrogel Type: Composed/functionalized hydrogel	Anti-inflammatory properties.	Atopic dermatitis (AD)	2,4-dinitrochlorobenzene (DNCB)-induced AD rat model	26
Nanostructured herbal formulations conditioned as appropriate hydrogel (HG) Type: Hydrogel as controlled delivery system	Antibacterial, antioxidant, anti-inflammatory and anti-acne activities.	Skin inflammation	Irritated rats and human volunteers	17
Poly(ethylene glycol) hydrogels presenting Arg- Gly-Asp (RGD) peptides and vascular endothelial growth factor Type: Composed/functionalized hydrogel	Promote cell adhesion and support various immune modulatory effects.	Skin inflammation	Skin wound in mice	30
Polypeptide hydrogel (QK-SF) constituted by silk fibroin (SF) and a vascular endothelial growth factor mimetic peptide KLTWQELYQLKYKGI (QK) Type: Composed/functionalized hydrogel	Proangiogenic and immunoregulatory activities.	Wound healing	Skin wound in mice	32

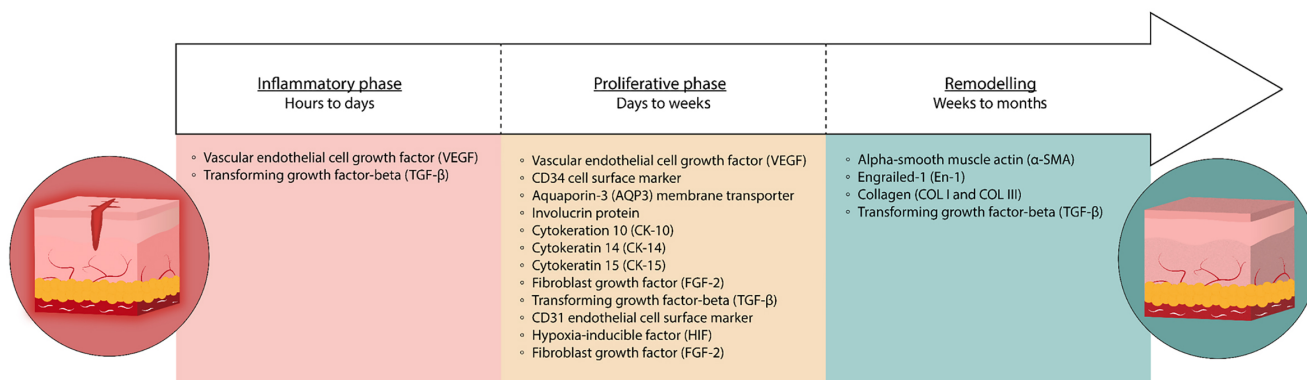


Figure 1. Factors involved in the different phases of cutaneous wound healing commonly investigated by IHC.

Another protein marker that was used for studying the effects of hydrogels on wound healing and scarring²⁷ is engrailed-1 (En-1), which is expressed by fibroblasts and leads to an over-deposition of the extracellular matrix and promotes the formation of scars in skin wounds.

An alternative target easily identifiable by IHC is collagen. Collagen type I represents the main structural element of the dermis that gradually replace collagen III, the major component of the granulation tissue. The immunolabelling for collagen I and collagen III was performed on FFPE rat or mouse skin samples using fluorescent (Alexa Fluor)²¹ or enzymatic (HRP/DAB or DAB) revealing systems^{28,33,37} in order to evaluate the ability of various kind of hydrogels to promote matrix remodelling during the regenerative process. In addition, Neves *et al.* demonstrated that polysaccharide-rich hydrogel formulation combined with photobiomodulation provides a concomitantly increase in the levels of collagen I, III and FGF-2, thus demonstrating that these combined treatments are effective in repairing the UV-induced photodamage in the skin of hairless mice.³³

Another biomarker widely studied is TGF- β that is involved in different processes of the normal tissue repair mechanisms, including inflammation, angiogenesis, fibroblast proliferation, collagen synthesis and deposition as well as matrix remodelling. An overexpression of TGF- β 1 was observed by Tawfeek *et al.* in wound treated with metformin hydrochloride hydrogel in skin samples, thus demonstrating the effective topical healing activity of this hydrogel type:⁵ in this work, a semi-quantitative analysis of the immunopositivity was performed according to the Remmele immunoreactive score.³⁸

Most of the markers mentioned above have also been used to evaluate the regenerative potential of hydrogels in the case of skin damages caused by burns or inflammatory states.^{20,26} For example Napimoga *et al.* examined the efficacy of the topical 15d-PGJ2-poloxamer 407 hydrogel in an rat model of atopic dermatitis: a significant reduction of the immunopositivity positivity for the transcription factor RORYt and TNF- α was observed after the topical treatment, suggesting that this hydrogel was able to improve atopic dermatitis through a suppression of the immune response.²⁶

Concluding remarks

This review summarizes the recent advances in the development of hydrogels as medical devices for skin repair and regeneration. The research on hydrogels has greatly increased in the last years because of their several advantages compared to the classical therapeutic approaches; in particular, the hydrogels can be loaded with cells or bioactive compounds having antimicrobial, anti-inflammatory, angiogenic or proliferative properties, thus providing an active contribution to the wound healing.

Notably, IHC proved to be a key technique to test the efficacy of hydrogels. In fact, to assess the actual ability of hydrogels to improve the healing process, different biomarkers specifically expressed in the different healing phases need to be detected in skin sample and IHC proved to be a versatile, relatively simple and affordable technique for their visualization *in situ*. Thus, the classical immunohistochemical techniques have become indispensable for the development of engineered biomaterials in the leading-edge research field of regenerative medicine.

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