

Role of Hyaluronic Acids and Potential as Regenerative Biomaterials in Wound Healing

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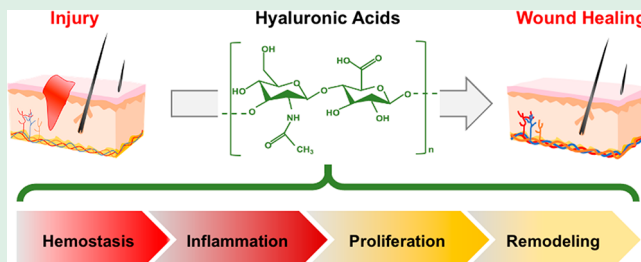
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ABSTRACT: The skin can protect the body from external harm, sense environmental changes, and maintain physiological homeostasis. Cutaneous repair and regeneration associated with surgical wounds, acute traumas, and chronic diseases are a central concern of healthcare. Patients may experience the failure of current treatments due to the complexity of the healing process; therefore, emerging strategies are needed. Hyaluronic acids (HAs, also known as hyaluronan), a glycosaminoglycan (GAG) of the extracellular matrix (ECM), play key roles in cell differentiation, proliferation, and migration throughout tissue development and regeneration. Recently, HA derivatives have been developed as regenerative biomaterials for treating skin damage and injury. In this review, the healing process, namely, hemostasis, inflammation, proliferation, and maturation, is described and the role of HAs in the healing process is discussed. This review also provides recent examples in the development of HA derivatives for wound healing.

KEYWORDS: complexity of healing process, hyaluronan, biological materials, regenerative medicine, combination therapy



1. INTRODUCTION

Wound healing is composed of four sequential but overlapping phases, namely, hemostasis, inflammation, proliferation, and remodeling (maturation) (Figure 1). The healing process proceeds efficiently in acute wounds (e.g., surgical incisions, traumatic injuries, abrasions, and superficial burns).¹ However, certain physiological states (e.g., age, infection, and large injury) or underlying medical conditions (e.g., ischemia, diabetes, and obesity) interfere with wound healing.² Consequently, the healing process is “stalled” at one of the phases, resulting in chronic (nonhealing) wounds. Chronic wounds occur along with complications such as inflammation, cell dysfunction, and impaired angiogenesis, which complicate medical care, increase treatment cost, and worsen the life quality of patients.³

Surgical debridement, antibiotics, and wound dressings remain the mainstay of therapeutic modalities for wounds.⁴ Strategies such as cytokine/growth factor therapy and cell therapy have been also developed for reparative and regenerative treatments.⁵ However, despite profound progress, a number of barriers limit the application of these approaches in wound healing. For example, these options are designed for targeting a single phase of the healing process, while synergistic effects are less studied.^{6,7} In addition, administration of cytokines and growth factors to the wound shows limited efficacy due to the lack of appropriate delivery systems.⁸ Healing efficacy of cell therapies is also dampened by the pathological state of donors, the onset time and duration of

treatment, and the administration dose and route.⁹ Thus, efficient and widely applied alternatives are highly desirable.

Hyaluronic acids [HAs, a glycosaminoglycan (GAG) of the extracellular matrix (ECM)] are widely distributed throughout the body; for example, ~50% of the total HAs are found within the skin.¹⁰ HAs are capable of maintaining hydration for space filling, improving viscosity of body fluids for lubrication of joints, and providing a framework for cell adhesion, proliferation, and migration. Recently, development of HA derivatives has demonstrated they have great potential as regenerative biomaterials for tissue repair and regeneration.¹¹ This review will describe the physicochemical and biological characteristics of HAs relevant to reparative and regenerative processes and discuss recent advances of HA derivatives for wound healing.

2. COMPLEXITY OF HEALING PROCESS

2.1. Hemostasis. Hemostasis occurs after the onset of a lesion to prevent bleeding from damaged blood vessels (Figure 1). When the endothelium of blood vessels is damaged, the underlying subendothelial matrix is exposed. The circulating

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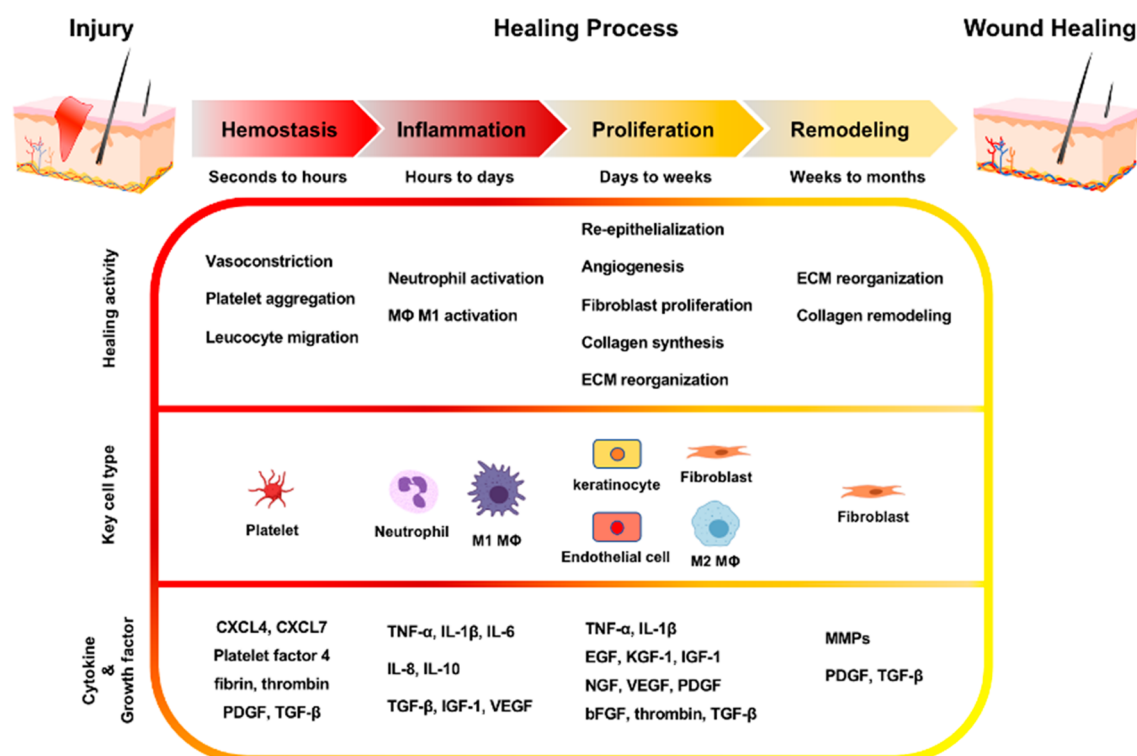


Figure 1. Wound healing process. It comprises hemostasis, inflammation, proliferation, and remodeling. In each phase, the cross-talk between distinct cell types and cytokines/growth factors exerts multiple biological activities for wound healing (C–X–C motif ligands 4 and 7, CXCL4 and CXCL7; platelet-derived growth factor, PDGF; transforming growth factor β , TGF- β ; tumor necrosis factor α , TNF- α ; interleukins 1 β , 6, 8, and 10, IL-1 β , IL-6, IL-8, and IL-10; insulin-like growth factor 1 = IGF-1; vascular endothelial growth factor = VEGF; epidermal growth factor = EGF; keratinocyte growth factor 1, KGF-1; nerve growth factor, NGF; basic fibroblast growth factor, bFGF; matrix metalloproteinases, MMPs; M Φ , macrophage).

platelets bind to the endothelium or subendothelial matrix *via* the interaction between the platelet receptors [e.g., integrin and glycoprotein VI (GPVI)] and the ECM adhesion molecules [e.g., P-selectin and von Willebrand factor (VWF)].¹² Subsequently, platelets are recruited into the site of injury and become activated to form an aggregation, namely, “platelet plug”. In addition, the activated platelets produce platelet-specific proteins (e.g., platelet factor 4, fibrin, and thrombin),¹³ which result in the constriction of blood vessels (vasoconstriction) and the initiation of coagulation cascades, facilitating the formation of blood clots.¹⁴ The formation of hemostatic plugs and clots stops the bleeding from the damaged blood vessels. Moreover, the activated platelets produce chemokines [e.g., C–X–C motif ligands 4 and 7 (CXCL4 and CXCL7)] and growth factors [e.g., platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β)].¹⁵ These components trigger the migration and activation of immune cells (e.g., neutrophils and macrophages) and residential skin cells (e.g., keratinocytes, endothelial cells, and fibroblasts), forming a provisional wound framework within the clots to promote later phases of the healing process.¹⁶

It is worth noting that certain conditions, such as venous insufficiency, diabetes, and thrombocytopenia, interfere with hemostasis and cause poor wound healing. Management of these conditions is critically important in assisting the healing process.¹⁷

2.2. Inflammation. In this phase, the innate immune system is activated to destroy the invading pathogens and remove the dead tissues, which sterilize the wound bed for the

growth of new tissues (Figure 1). As the dominant immune cells at the early stage of inflammation, neutrophils are recruited into the wound from the bloodstream within hours after hemostasis.¹⁸ Microbes and debris are engulfed by neutrophils *via* phagocytosis and are subsequently degraded by reactive oxygen species (ROS) and proteases [e.g., serine proteases and matrix metalloproteinases (MMPs)] within the phagocytic vacuole.¹⁹ In addition, neutrophils produce cytokines [e.g., tumor necrosis factor α (TNF- α), interleukin 1 β (IL-1 β), and IL-6], which can augment inflammatory responses to sterilize the wound.²⁰ In normal healing process, neutrophils proceed apoptosis shortly after accomplishing their function.²¹ However, the pathogenesis such as infections and autoimmune diseases triggers necrosis of neutrophils.²² The integrity of the cell membrane is maintained in neutrophils during apoptosis, while necrosis causes the release of ROS and proteases into the extracellular milieu, which can damage host tissues and degrade growth factors, growth factor receptors, and the ECM, causing the retardation in wound healing.²³

Concomitantly with the recruitment of neutrophils, the circulating monocytes also arrive at the wound area and differentiate into macrophages.²⁴ As the dominant immune cells at the late stage of inflammation, macrophages phagocytose pathogens, and debris further sterilize the wound, facilitating tissue repair and regeneration in the proliferation phase.²⁵ In addition, macrophages recognize characteristic alterations of apoptotic neutrophils (e.g., upregulation of phosphatidylserine, a fatty substance) and remove these cells *via* phagocytosis (also termed efferocytosis).²⁶ The phagocytosis of apoptotic neutrophils induces the

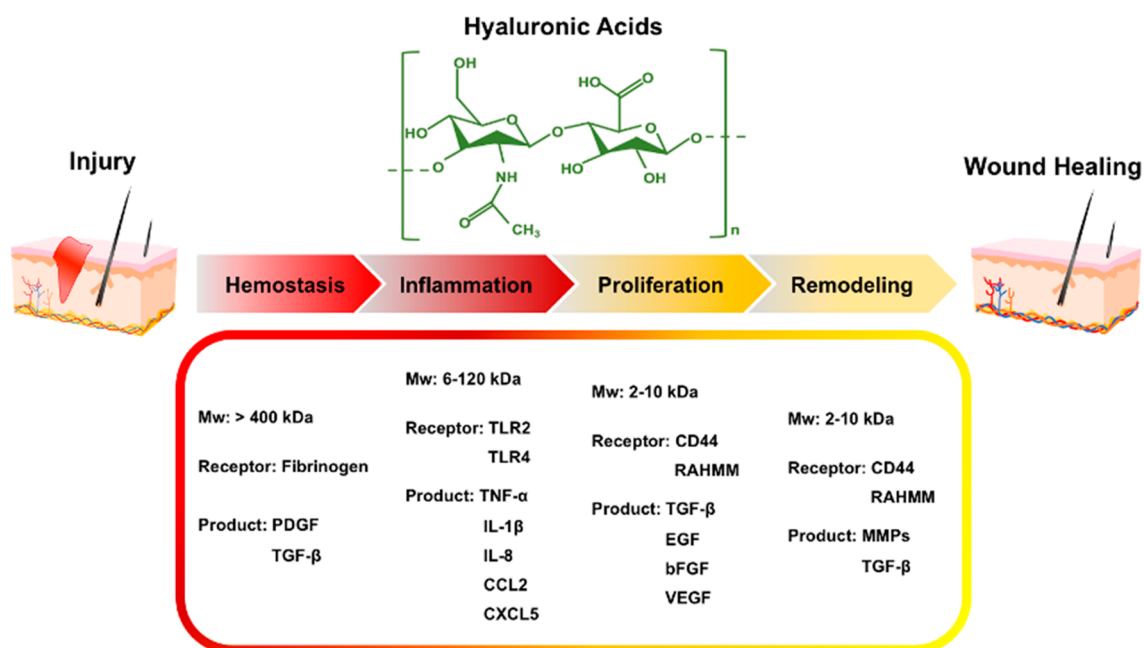


Figure 2. HAs and wound healing. HAs are produced by polymerization of *N*-acetyl-D-glucosamine and D-glucuronic acid via a β -(1–3)-glucuronidic bond. HAs with different MWs bind to HA receptors on skin and immune cells for producing cytokines, chemokines, and growth factors, which can directly and indirectly regulate wound healing (platelet-derived growth factor, PDGF; transforming growth factor β , TGF- β ; tumor necrosis factor α , TNF- α ; interleukins 1 β and 8, IL-1 β and IL-8; C–C motif chemokine 2, CCL2; C–X–C motif ligand 5, CXCL5; epidermal growth factor, EGF; basic fibroblast growth factor, bFGF; vascular endothelial growth factor, VEGF; matrix metalloproteinases, MMPs; toll-like receptors 2 and 4, TLR 2 and TLR4; receptor for HA-mediated motility, RHAMM).

conversion of macrophages from pro-inflammatory M1 phenotype to an anti-inflammatory M2 one.²⁷ M2 or M2-like macrophages release immunosuppressive cytokines (e.g., IL-10) for the resolution of inflammation.²⁸ M2 or M2-like macrophages also produce growth factors including TGF- β , PDGF, insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF).²⁹ These components enhance cell proliferation, angiogenesis (the formation of new blood vessels) and the formation of granulation tissue,³⁰ supporting subsequent phases of the healing process. Notably, the inefficient clearance of neutrophils and the persistence of M1 macrophages without conversion to M2 phenotype can cause acute wounds to be chronic and halt the healing process.^{31–33}

It is worth noting that the heterogeneity and plasticity of neutrophils and macrophages are not fully characterized, and the role of potential stimuli to the resolution and phenotypic switch associated with macrophages is not completely understood.^{34,35} Therefore, future studies are needed to solve these issues, in order to improve therapeutic benefits for chronic wounds. In addition, mast cells, T and B lymphocytes, are also involved in the late stage of inflammation, which has been discussed elsewhere.^{36,37}

2.3. Proliferation. The proliferation phase is composed of three stages, namely, re-epithelialization (the closure of a wound's surface), angiogenesis, and the formation of granulation tissue (Figure 1).³⁸

Re-epithelialization is mainly accomplished by keratinocytes (they represent ~90% of the cells of the epidermis).³⁹ Keratinocytes can be released from the wound vicinity by the ECM enzymes (e.g., collagenases and elastases) and accumulate on the wound bed.⁴⁰ In addition, the contact inhibition and physical tension at the desmosome and hemidesmosome (they are attachment sites at the cell surface

for cell–cell and cell–substrate connections)⁴¹ are impaired in wound edges, which result in the cytoskeletal elongation and reorganization, facilitating the migration of keratinocytes into the wound bed.⁴⁰ The proliferation of keratinocytes is regulated by growth factors such as keratinocyte growth factor 1 (KGF-1), epidermal growth factor (EGF), nerve growth factor (NGF), and IGF-1.⁴² In addition, epithelial stem cells at the hair follicle and sweat gland are also involved in re-epithelialization, which has been described elsewhere.⁴³

Angiogenesis is regulated by growth factors [e.g., VEGF, PDGF, and basic fibroblast growth factor (bFGF)] and the serine protease thrombin.⁴⁴ These components stimulate endothelial cells of the existing vessels, and the activated endothelial cells release proteolytic enzymes to dissolve the basal lamina.³⁸ Subsequently, the activated endothelial cells migrate toward the angiogenic stimuli and proliferate inside the wound, leading to the formation of a microvascular network (also known as “sprout”).⁴⁵ The sprouts differentiate into blood vessels, and the vessel wall is stabilized by pericytes and smooth muscle cells.⁴⁶

In the late stage of proliferation, the provisional wound framework established during hemostasis is substituted by the formation of granulation tissue. The granulation tissue is characterized as a type of connective tissue that consists of fibroblasts, keratinocytes, macrophages (mainly M2 phenotype), blood vessels, and collagens.⁴⁷ The ECM formation within the granulation tissue is highly regulated by fibroblasts.⁴⁸ Following the stimulation of cytokines (e.g., TNF- α and IL-1 β) and growth factors (e.g., TGF- β , PDGF, and bFGF), fibroblasts migrate from the nearby dermis and become activated inside the damaged site.⁴⁹ The activated fibroblasts generate MMPs to degrade the provisional wound framework⁵⁰ and meanwhile produce the ECM components such as collagens (mainly type III collagen), GAGs, and

proteoglycans, for the formation of granulation tissue.⁴⁰ In addition, the activated fibroblasts also produce TGF- β , KGF-1 and bFGF, which regulate other types of cells within the granulation tissue for tissue repair and scarring in the remodeling phase.⁵¹

Notably, the angiogenesis is not fully accomplished in the proliferation phase; therefore, the granulation tissue is highly angiogenic and can be easily damaged.⁵² In addition, chronic wounds are associated with the failure in the formation of granulation tissue. For example, ROS is excessively produced in the wound of patients who have the deficiency of ROS detoxifying enzymes,⁵³ and the overproduction of ROS generates a large amount of MMPs that can degrade the granulation tissue and retard the healing process.⁵⁴

2.4. Remodeling. The remodeling phase initiates at the same time when the granulation tissue is formed. In this phase, the components inside the ECM undergo significant changes. For example, type III collagen, which is an immature collagen generated from fibroblasts during the proliferation phase, is replaced by type I collagen that has a higher tensile strength.⁵⁵ In addition, fibroblasts are differentiated into myofibroblasts in response to TGF- β during the formation of granulation tissue.⁵⁵ Myofibroblasts promote the wound contraction through the attachment to collagens and alleviate the scar formation on the wound surface.⁵⁶ Furthermore, the formation of a vascular network is accomplished, the blood flow at the wound site declines, and the metabolic activities slow and finally cease.⁵⁷

Of note, the excessive scarring or fibrosis (e.g., hypertrophic scars and keloid) remain major issues in the remodeling phase.¹⁷ The overproduction of cytokines or growth factors causes a large amount of granulation tissue, which will exacerbate the scar formation.⁵⁸ The failure in the remodeling of type III collagen to type I also causes excessive scar formation.⁵⁶ However, no satisfactory therapeutic strategy is available to date for treating scar or fibrosis.

As described above, the interplay between distinct cell types and numerous factors (e.g., cytokines and growth factors) is well-orchestrated during the normal healing process. However, when the aberration occurs to this interplay, the healing process is “stalled” at one of the phases, leading to chronic (nonhealing) wounds. Therefore, an improved understanding in the pathophysiological conditions underlying delayed healing will foster the development of efficient and widely applied therapeutic strategies.^{6–9} Recently, a variety of reparative and regenerative biomaterials have been developed to promote wound healing by targeting multiple phases associated with the impaired processes.^{59–62} Among these, HAs have demonstrated great potential for treating skin damage and injury, which will be discussed in the following sections.

3. DEVELOPMENT OF HYALURONIC ACID DERIVATIVES FOR WOUND HEALING

3.1. Roles of Hyaluronic Acids in Wound Healing. The naturally occurring HAs are a biocompatible and biodegradable GAG (an anionic long linear polysaccharide) (Figure 2). As an essential ECM component, HAs are able to promote cell differentiation, proliferation, adhesion, and migration during organism development (ontogenesis) and regeneration.⁶³ HAs are produced inside cells by polymerization of *N*-acetyl-D-glucosamine and D-glucuronic acid via a β -(1–3)-glucuronidic bond using HA synthases (HAS, a group of membrane-bound

enzymes).⁶⁴ To date, three types of HAS have been identified, namely, HAS1, HAS2, and HAS3. HAs with MWs over 2×10^3 kDa, from 2×10^2 to 2×10^3 kDa, and from 1×10^2 to 1×10^3 kDa, and are produced by HAS2, HAS1, and HAS3, respectively.⁶⁵ HAs have an efficient turnover rate (e.g., half-life of less than 1 day within the skin)⁶⁶ and are catabolized at the extracellular milieu by two main pathways: (1) They can be hydrolyzed by hyaluronidases or fragmented by oxygen free radicals and subsequently are removed from the lymphatic system;⁶⁷ (2) they can be taken up by neighboring cells and transported into endosomes/lysosomes for enzymatic degradation.⁶⁸ It is well-established that the biological activities of HAs are highly reliant on molecular weight (MW).^{69,70} HAs with different MWs bind to a number of HA receptors, mainly including CD44, a receptor for HA-mediated motility (RHAMM), and toll-like receptors 2 and 4 (TLR2 and TLR4) (Figure 2).¹⁰ The interaction between HAs and HA receptors mediates a variety of intracellular signaling pathways for the regulation of wound healing, which has been previously described.^{10,63}

At the hemostasis phase, a large amount of HAs with high MW (HMW-HAs, ranging from 4×10^2 to 2×10^4 kDa) is produced from platelets within the wound (Figure 2).⁷¹ HMW-HAs bind to fibrinogen (also known as a clotting factor I; it circulates in the blood), resulting in efficient clot formation.⁷² Because HMW-HAs are very hydrophilic, they are saturated with fluids to swell around the wound (termed edema). The edema forms a temporary framework for the access of immune cells to the injured area.⁷¹

At the inflammation phase, HMW-HAs are catabolized into HAs with low MW (LMW-HAs, less than 120 kDa) at the inflamed sites (Figure 2).⁷³ HMW-HAs demonstrate immunosuppressive and antiangiogenic features, while LMW-HAs are generally considered immunostimulatory and pro-angiogenic.⁷⁴ LMW-HAs bind to TLR2 and TLR4 for the production of cytokines (e.g., TNF- α , IL-1 β , and IL-8) and chemokines [e.g., CXCL5 and C-C motif chemokine 2 (CCL2)], which promote the infiltration, activation, and maturation of immune cells.⁷⁵ Although inflammation is indispensable for successful wound healing, a persistence in this phase will cause acute wounds to become chronic (see section 2.2). Therefore, when the healing process moves forward, LMW-HAs will be catabolized into HA oligomers (O-HAs, ~6–20 monomers), which reduce the inflammatory response but improve the proliferative activity.⁷¹

At the proliferation phase, O-HAs alleviate the inflammation, enhance the re-epithelization, foster the angiogenesis, and improve the formation of granulation tissue (Figure 2). They bind CD44 and RHAMM to mediate the production of TGF- β , EGF, bFGF, and VEGF, resulting in the recruitment, maturation, and activation of keratinocytes,⁷⁶ endothelial cells,⁷⁷ and fibroblasts.⁷¹ For example, O-HAs stimulate endothelial cells and fibroblasts for synthesis and deposition of type III collagen within the wound, resulting in the formation of a new collagen matrix.^{78,79}

At the final phase of the healing process, O-HAs interact with CD44 and RHAMM to produce MMPs and TGF- β , which promote the differentiation of fibroblasts into myofibroblasts, facilitating the maturation of collagens (type I) for the ECM remodeling (Figure 2).⁷⁵ It has been reported that the accumulation of a disorganized collagen matrix is significantly inhibited in fetal wounds, resulting in scarless healing.⁸⁰ It is likely due to the fact that HAs in fetal wounds

display a higher level and longer period than those found in adult wounds.⁸⁰ Thus, strategies that promote a prolonged presence of a HA-rich matrix provide great potential for scarless repair and regeneration.⁸¹

In addition, HAs have also demonstrated antibacterial and -fungal activities, which have been previously discussed.^{82,83} For example, the antimicrobial effects of HMW-HA (MW = 1837 kDa) were assessed using 11 bacterial and 4 fungal strains (the representative of clinically relevant microbes).⁸² Results show that the growth of these microbes was affected by HMW-HA at different levels in a dose-dependent manner, which was likely due to (1) the water-retaining capacity of HAs that may delay or disrupt the growth of microbes and (2) the interactions between HAs and pathogen surface moieties to inhibit bacterial colonization.⁸² These results suggest that HAs may be a defense against pathogens in the wound area, which is favorable for the healing process (particularly at early stages).

3.2. Preparation and Characterization of Hyaluronic Acid Derivatives. Commercial HAs are traditionally manufactured by the extraction from animal tissues or by the fermentation of natural microorganisms.⁸⁴ However, impurities and toxins caused by animal tissues or pathogenic microbes are a concern for biomedical application of HAs. To address these issues, commercial HAs can be obtained from nonpathogenic and endotoxin-free recombinant microbes.⁸⁵ However, the large-scale production of HAs using genetically modified microbial hosts is limited due to the high cost of raw materials. In addition, it is difficult to precisely control the yield and MWs of HAs using the aforementioned approaches. Future work needs be carried out to understand intracellular metabolic pathways underlying the biosynthesis of HAs as well as to optimize the strategies of metabolic engineering (see discussion in more detail),^{86,87} which will potentially enhance the quality control in the yield and uniformity of HAs.

3.2.1. Chemical Modification. The chemical methods and functionalization techniques that facilitate the development of HA derivatives with distinct biological and physicochemical properties have been substantially investigated (Figure 3).^{88,89} The hydroxyl (-OH) and carboxyl (-COOH) groups of HAs are preferred for chemical modification, achieving two types of HA derivatives, namely, conjugated and cross-linked HAs (Figure 3).^{88,89} HA conjugation is generated by grafting the monofunctional moiety to the single backbone, while HA cross-linking is produced by grafting bi- or polyfunctional groups between different chains (Figure 3). The conjugation allows (1) the decoration of functional groups to HAs achieving HA-based vehicle systems with enhanced drug delivery features relative to unmodified HAs and (2) the incorporation of therapeutic agents to HAs achieving HA-drug conjugates. The cross-linking improves the physicochemical features of HAs for a reduced degradation rate, prolonged resident time, and controlled drug release.⁹⁰

The hydroxyl group can be modified to generate HA derivatives with ethers (using epoxides, divinyl sulfone, and ethylene sulfide under alkaline conditions),^{91–93} hemiacetals (using glutaraldehyde in acetone–water solutions),⁹⁴ esters (using octenyl succinic anhydride and methacrylic anhydride in basic water),^{95,96} and carbamates (using cyanogen bromide in alkaline aqueous environments).⁹⁷ For example, a HA derivative (namely, HA-MA) was produced by chemically functionalizing the hydroxyl group of HAs (MW = 1000 kDa) using methacrylic anhydride (MA) in basic water (pH = 8) at

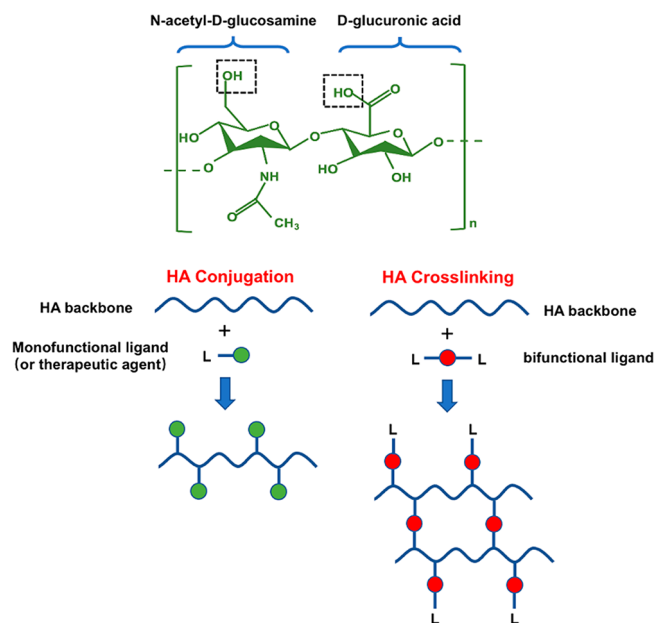


Figure 3. Schematic for chemical modification of HAs. The hydroxyl (-OH) and carboxyl (-COOH) groups of the HA disaccharide unit are preferred for chemical modification. Monofunctional ligands (or wound healing agents) and bi-/polyfunctional groups can be used for the production of conjugated and cross-linked HAs, respectively.

4 °C for 2 days.⁹⁸ Following the stimulation of UV irradiation (365 nm for 20 min), a hydrogel was polymerized from a mixture of HA-MA and N-hydroxyethylacrylamide-modified dextran. The resultant hydrogel was used for co-encapsulation of β -cyclodextrin containing resveratrol (an anti-inflammatory substance) and poly(ethylenimine) containing plasmid DNA (VEGF protein). This hydrogel system could accelerate the burn wound healing in rats by means of suppressing inflammation responses and enhancing blood vessel formation.⁹⁸

The carboxyl group of HAs can be esterified and amidated.⁸⁸ The esterification may be carried out by alkylating the HA carboxyl group using alkyl halides (e.g., alkyl iodides or bromides) and tosylate activation.^{99,100} The esterification of HAs may also be achieved by activating the carboxyl group using diazomethane.^{101,102} These reactions must be carried out in organic solvents using either the acidic form or the tetrabutylammonium (TBA) salt of HAs. In addition, the esterification may be exerted using epoxides in aqueous solutions.¹⁰³ For example, a HA derivative (namely, HA-ADH) was generated by chemically conjugating the carboxyl group of HAs (MW = 200 kDa) in the DMSO/water solution (v:v, 1:1) using adipic dihydrazide (ADH), and the reaction was performed until the pH value was adjusted to ~ 7.0 .¹⁰⁴ A hydrogel was subsequently formed from a mixture of HA-ADH and oxidized dextran under slight stirring, which was used for the encapsulation of gelatin microspheres containing sanguinarine (SA, a drug for antibacterial and anti-inflammatory properties).¹⁰⁴ Consequently, the hydrogel formulation significantly improved the wound healing in a rat burn infection model, which was accompanied by enhanced re-epithelialization and ECM remodeling, reduced inflammatory responses, and decreased bacterial activities.¹⁰⁴ Moreover, the amidation on the carboxyl group of HAs may be achieved using carbodiimides,¹⁰⁵ carbonyldiimidazole,¹⁰⁶ 2-chloro-1-methylpyridinium iodide (CMPI),¹⁰⁷ and 2-chlorodimethoxy-1,3,5-

triazine (CDMT).¹⁰⁸ For example, a HA derivative (namely, HA-AEMA) was produced by chemically activating the carboxyl group of HAs (MW = 90–110 kDa) using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide, *N*-hydroxysuccinimide, and aminoethyl methacrylate (AEMA).¹⁰⁹ A hydrogel system was subsequently developed from a mixture of HA-AEMA and methacrylated methoxy poly(ethylene glycol) (MA-mPEG) for delivery of chlorhexidine diacetate (an antimicrobial agent), which demonstrated quick hemostasis and reduced wound infection, leading to efficient wound healing in the rat full-thickness wound model.¹⁰⁹

In addition to hydroxyl and carboxyl groups, the *N*-acetyl group of HAs may be modified by deacetylation, which can recover an amino group. The deacetylated HA amine can be conjugated with an acid to form the amide bond.¹¹⁰ The deacetylated amino moiety can also react with -COOH group of HAs, potentially resulting in a cross-linked hydrogel. However, it is worth noting that the deacetylation is generally carried out under intensive experimental conditions, which possibly cause the degradation of HAs.¹⁰⁵ In addition, the HA structure may be dramatically changed by deacetylation, which impairs the biological properties of parent HAs.¹¹¹ Therefore, the chemical modification must maintain the biocompatibility, biodegradability, and mucoadhesivity of HAs.

3.2.2. Physicochemical Characterization. The chemical structure of HA derivatives is commonly characterized using nuclear magnetic resonance spectroscopy (NMR).^{112–114} For example, unidimensional ¹H NMR spectrometry can be applied to identify the conjugated compound(s) and quantify the degree of modification within HAs.¹¹⁵ However, this technique is not capable of confirming the type of covalent bonds and therefore requires complementary characterization. Infrared spectroscopy (IR) as a complementary characterization technique provides the information on covalent bonds which are formed during the modification of HAs.^{116,117}

The physical morphologies/structures play significant roles for the development of reparative and regenerative biomaterials.¹¹⁸ The physical morphology of HAs (a dried form) may be evaluated using scanning electron microscopy (SEM).^{119,120} The SEM results demonstrate that native or conjugated HAs have a fibrous and irregular two-dimensional (2D) morphology, while cross-linked HAs possess a porous 3D structure in which therapeutic agents and/or living cells may be encapsulated.^{119,120} Atomic force microscopy (AFM) can provide the information on physical structure (e.g., topography and roughness) of HAs in aqueous environments.¹¹⁶ In addition, dynamic light scattering (DLS) techniques can be used to measure hydrodynamic diameter and size distribution of HAs within aqueous conditions.^{121,122}

The physical properties such as viscosity, shear-thinning, swelling, and mechanical strength are also used for characterizing biomaterials, which may affect their reparative and regenerative performance in tissue engineering.^{123,124} When the MW of HAs is increased to >10³ kDa, the aqueous solutions form strengthened networks, resulting in gradually elevated viscosity.¹²⁵ In addition, the viscosity of HA aqueous solutions, due to the polyelectrolyte nature of HAs, is affected by ionic strength, pH, and temperature.^{125,126} The variation of these parameters in HA aqueous solutions causes the repulsive or attractive forces between and within the HA chains, significantly taking effect on the viscosity.^{125,126} For example, the backbone of HAs is degraded by hydrolysis in aqueous solutions with the pH values < 4 or > 11, which cause the loss

of viscosity.¹²⁷ Furthermore, HA aqueous solutions are considered shear-thinning (or pseudoplastic); when the shear rate increases, the viscosity declines, which is due to the disruption of hydrogen bonds and hydrophobic interactions between and within HAs under the shear stress.¹²⁸ The swelling property is an important indicator of the water-retaining capacity of hydrogels.¹²⁹ The hydrogels with stronger HA cross-linking correspond to the lower swelling rate and the less water uptake capacity.¹³⁰ It has been reported that the swelling measurements were applied to assess the cross-linking efficacy of four linkers on HAs, namely, glutaraldehyde, divinyl sulfone, carbodiimide, and bisepoxide.⁹⁴ Results indicated that glutaraldehyde and divinyl sulfone were more efficient cross-linkers than the others.⁹⁴ In addition, the mechanical strength (an ability to withstand an applied stress without the plastic deformation) is an important parameter for the preparation and application of biomaterials.¹³¹ With respect to wound healing, conjugated HA encapsulated inside a formulation or cross-linked HA hydrogels can be applied as dressings (or bandages) onto the injured area.¹³² Different experimental approaches based on Young's modulus, namely, tensile test, compression test, and indentation test,^{133–135} can be applied to investigate the mechanical strength of HA-based dressings, ensuring them to possess excellent reliability of the graft. For example, a serial of HA hydrogels were produced from a mixture of methacrylated HA (MeHA; MW = 50, 350, and 1100 kDa) and PEGylated dimethacrylate using a UV photopolymerizing method.¹³⁶ The compressive moduli of these HA hydrogels were measured using a dynamic mechanical analyzer. Results from stress *vs* strain plots indicate that the modulus was well-correlated with the cross-linking density within HA hydrogels. The moduli were significantly elevated when the concentration was increased for each of MeHA MW; for example, the modulus was 12 kPa for 2 wt % 50 kDa MeHA, while the value was increased to 100 kPa when the concentration rose to 20 wt %.¹³⁶ Consequently, when fibroblasts were loaded, cell viability was significantly reduced in the HA-based network with the compressive modulus of >100 kPa, which was likely due to the limitation in the transport of nutrients into the hydrogels with high cross-linking density.¹³⁶

As compared to native or conjugated HAs, the cross-linked HAs may be designed to form injectable hydrogel systems *in situ* with favorable physicochemical properties such as reduced degradation rate, longer residence time, and improved release characteristics.^{90,137,138} Injectable HA hydrogels improve the porosity and swelling properties, which facilitate the exchange of nutrients and the encapsulation of therapeutic agents and/or living cells (e.g., fibroblasts, microvascular endothelial cells, and mesenchymal stem cells), promoting host cells, over time, to generate their own ECM and replace the degraded hydrogels.¹³⁹ Injectable HA hydrogels achieve the proper gelation and release profiles for the particular application of interest by means of selecting efficient cross-linkers and adjusting cross-linking kinetics, which have been recently discussed elsewhere.^{140–142}

3.2.3. Biological Assessment. Due to the favorable physicochemical and biological properties, novel HA derivatives have been developed for wound healing. The *in vitro* and *in vivo* experimental strategies have been utilized to assess the healing efficacy of HA derivatives.

For example, the *in vitro* anti-inflammatory properties can be assessed using primary or cultured macrophages where the

Table 1. Recent *In Vivo* Studies on HA Derivatives in Wound Healing, Including Formulation Strategy, Molecular Weight, Conjugation, Animal Model, and Healing Mechanism

formulation	MW	conjugation	animal model	healing mechanism	ref
O-HA ointment	2–10 units	none	streptozotocin-induced diabetic rats with full-thickness wound	promoted proliferation, migration and angiogenesis	146
curcumin-grafted HA-modified pullulan polymers	5.4 kDa	curcumin, pullulan	rats with full-thickness wound	high swelling, fast hemostasis, good cyto- and hemocompatibility, antimicrobial and antioxidant properties	147
agarose-grafting HA scaffolds	9 kDa	agarose	mice with full-thickness wound	upregulation of type III collagen	144
alginate-based BHA-containing gel	40 kDa	butyrylation	rats with full-thickness wound	downregulation of TNF- α upregulation of MAPK pathway	143
aminoethyl methacrylate HA and methacrylated methoxy poly(ethylene glycol) hybrid hydrogels and chlorhexidine diacetate-loaded nanogels	90–110 kDa	aminoethyl methacrylate, methacrylated methoxy polyethylene glycol	mice with full-thickness wound	downregulation of NF- κ B pathway downregulation of TNF- α , IL-1 β , IL-6, and MCP-1	109
silk fibroin/HA/sodium alginate composite scaffolds	200 kDa	silk fibroin	rats with full-thickness burn	favorable wound healing environment and improved collagen deposition	148
hydrogels containing quaternized chitosan, oxidized HA, and HA-hydrazide	200 kDa	hydrazide, oxidation	rats with full-thickness wound	upregulation of TGF- β 1	149
dextran-HA hydrogels enriched with sanguinarine incorporated into gelatin microspheres	200 kDa	dextran, sanguinarine	rats with full-thickness wound	downregulation of TNF- α , IL-1 β , and IL-6 upregulation of TGF- β 3	104
poly(dimethylsiloxane)-based patch formulation containing epidermal growth factor-conjugated HA	200 kDa	epidermal growth factor	diabetic rats with full-thickness wound	downregulation of TNF- α enhanced proliferation and migration of keratinocytes	151
hydrogel formulation prepared by cross-linking HMW-HAs (200 kDa)-tyramine (HMW-HA-Tyr) with collagen L-hydroxybenzoic acid under the stimulation of horseradish peroxidase and H ₂ O ₂	200 kDa	tyramine, collagen L-hydroxybenzoic acid	rats with full-thickness wound	improved proliferation of skin cells and secretion of VEGF	150
layer-by-layer membranes containing chitosan, alginate and dopamine-HA	595 kDa	dopamine	rats with full-thickness wound	enhanced cell adhesion and proliferation	152
Hydrogels generated by chemically modified HA, dextran and β -cyclodextrin containing resveratrol and VEGF plasmid	1000 kDa	dextran	rats with full-thickness wound	downregulation of TNF- α and IL-1 β	98
HA and ECM fiber scaffolds	1500–1800 kDa	none	mice with full-thickness wound	accelerated granulation tissue formation, angiogenesis, and re-epithelialization	153

activity of the inflammatory signaling pathways [e.g., mitogen-activated protein kinases (MAPK) and nuclear factor- κ B (NF- κ B) pathways] is evaluated *via* reverse transcriptase-polymerase chain reaction (RT-PCR) to quantify the mRNA level and *via* Western blotting or enzyme-linked immunosorbent assay (ELISA) to measure the protein level.^{143,144} The repolarization of M1 macrophages to M2 can also be evaluated using flow cytometry and immunofluorescent microscopy to confirm the anti-inflammatory activity.¹⁴⁵ In addition, the *in vitro* proliferative properties can be investigated in cultured cell lines [e.g., human umbilical vein endothelial cells (HUVECs) and 3T3 cells (fibroblast-like cells)] using the migration (scratch) and tube formation assays.^{143,144,146} Furthermore, the *in vitro* remodeling characteristics can be evaluated using 3T3 cells whereby the level of secreted collagens I and III are measured using ELISA.¹⁴⁴

The *in vivo* wound healing efficacy can be assessed using a variety of animal models including full-thickness wound or burn rodents with certain physiological states (e.g., age) or underlying medical conditions (e.g., diabetes).^{146,147} The wound closure efficacies are monitored, and the wounds can be collected at different time points for the following experiments: (1) the hematoxylin–eosin (H&E), Masson's trichrome, and immunohistochemical staining experiments to confirm histopathological changes;^{148,149} (2) RT-PCR, Western blotting, and ELISA to determine the activity of inflammation, proliferation, and remodeling pathways.^{150,151}

3.3. Recent Advances of Hyaluronic Acid Derivatives for Wound Healing. Recent *in vivo* studies of HA derivatives are summarized in Table 1, and selected examples are discussed below on the basis of MWs of HAs (Figures 4 and 5).

3.3.1. O-HAs. Wang *et al.* have produced small HA fragments (ranging from 4 to 20 monomers) using HMW-HAs by hyaluronidase, and the resultant O-HAs were loaded into a slow-releasing ointment using the emulsification method.¹⁴⁶ Results show that the O-HA ointment effectively exerted the angiogenic activities (e.g., proliferation, migration, and tube formation) of HUVECs. These mainly resulted from the enhancement of the MAPK pathway, which was confirmed by the increment of TGF- β and phosphorylated Src and ERK (two proteins in the MAPK pathway).¹⁴⁶ When the O-HA ointment was topically applied to the excisional full-thickness wounds of streptozotocin-induced diabetic rats, it was capable of significantly improving the wound healing relative to the blank formulation.

3.3.2. LMW-HAs. It has been reported that several LMW-HAs were prepared by partial N-deacetylation *via* hydrazinolysis or NaOH treatment of HMW-HAs, and the amine group of LMW-HAs was subsequently acetylated with acyl anhydrides.¹⁵⁴ Among these, N-butyrylated LMW-HA derivative (termed BHA, ~40 kDa) significantly suppressed the NF- κ B pathway, leading to the downregulation of TNF- α , IL-6, and IL-1 β , *in vitro*¹⁵⁴ and *in vivo*.¹⁴³ This indicated that BHA is different from classic LMW-HAs that are generally considered immunostimulatory. In addition, BHA significantly improved the tube formation and migration of HUVECs,¹⁴³ which were mainly due to the enhancement of the MAPK pathway. When the alginate-based BHA-containing formulation was topically applied to rats with excisional full-thickness wounds, it was able to efficiently promote the formation of a new vascular system, modulate the inflammatory response, and improve the remodeling of type III collagen to type I.¹⁴³ These suggest

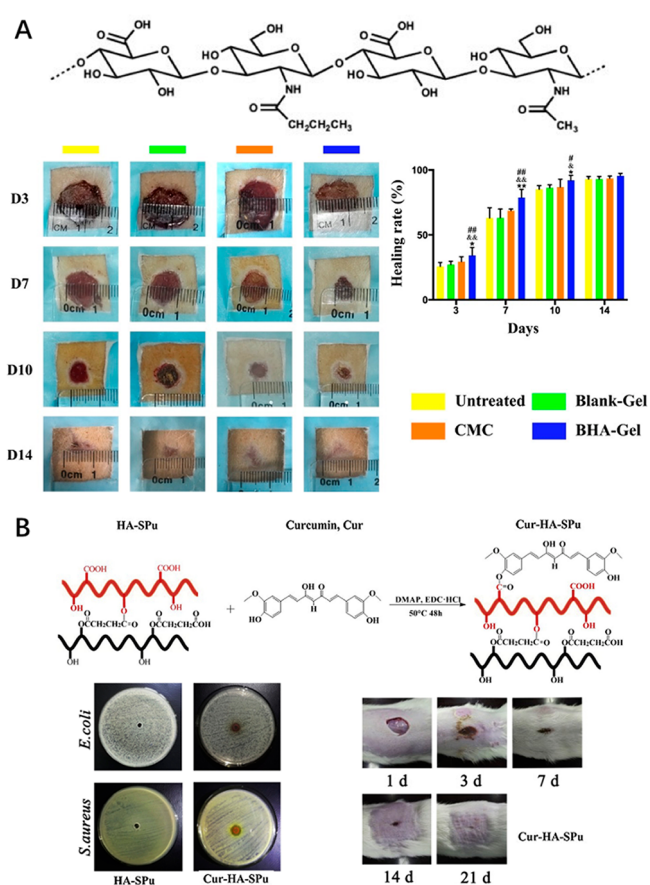


Figure 4. (A) LMW-HA derivative: modulating inflammation, enhancing epithelialization and angiogenesis, and remodeling collagens for excisional wound healing. Adapted with permission from ref 143. Copyright 2019 The Authors under Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>), published by MDPI, Basel, Switzerland. (B) Curcumin-grafted LMW-HA derived pullulan polymer. Antibacterial and antioxidant activities were achieved for wound healing. Adapted with permission from ref 147. Copyright 2020 Elsevier.

that BHA could fulfill the inflammation, proliferation, and maturation phases. Notably, the BHA formulation significantly improved therapeutic efficacy relative to a commercial wound care product (CHITIN), providing great promise for clinical translation to wound healing (Figure 4A).

In addition, three curcumin (Cur)-conjugated HA-grafted pullulan (sPu) polymers (Cur-HA-sPu) were produced with different ratios (Cur:HA-sPu = 1:5, 1:10, and 1:15) to form the film formulation for wound healing (Figure 4B).¹⁴⁷ The chemical structure of the resultant polymers was confirmed using ¹H NMR, IR, and differential scanning calorimetry (DSC).¹⁴⁷ These polymers demonstrated favorable *in vitro* properties, including less cytotoxicity, improved cell proliferation, and robust antibacterial and antioxidant activities.¹⁴⁷ As a result, they significantly accelerated the healing of full-thickness wounds in rats as compared to a commercial bandage (Figure 4B).

3.3.3. HMW-HAs. Recently, Kim *et al.* have reported that epidermal growth factor (EGF) was conjugated with HMW-HAs (200 kDa), and the HMW-HA-EGF conjugate was subsequently encapsulated by a poly(dimethylsiloxane) (PDMS, Sylgard 184)-based patch formulation.¹⁵¹ The resultant formulation enhanced proliferation and migration of

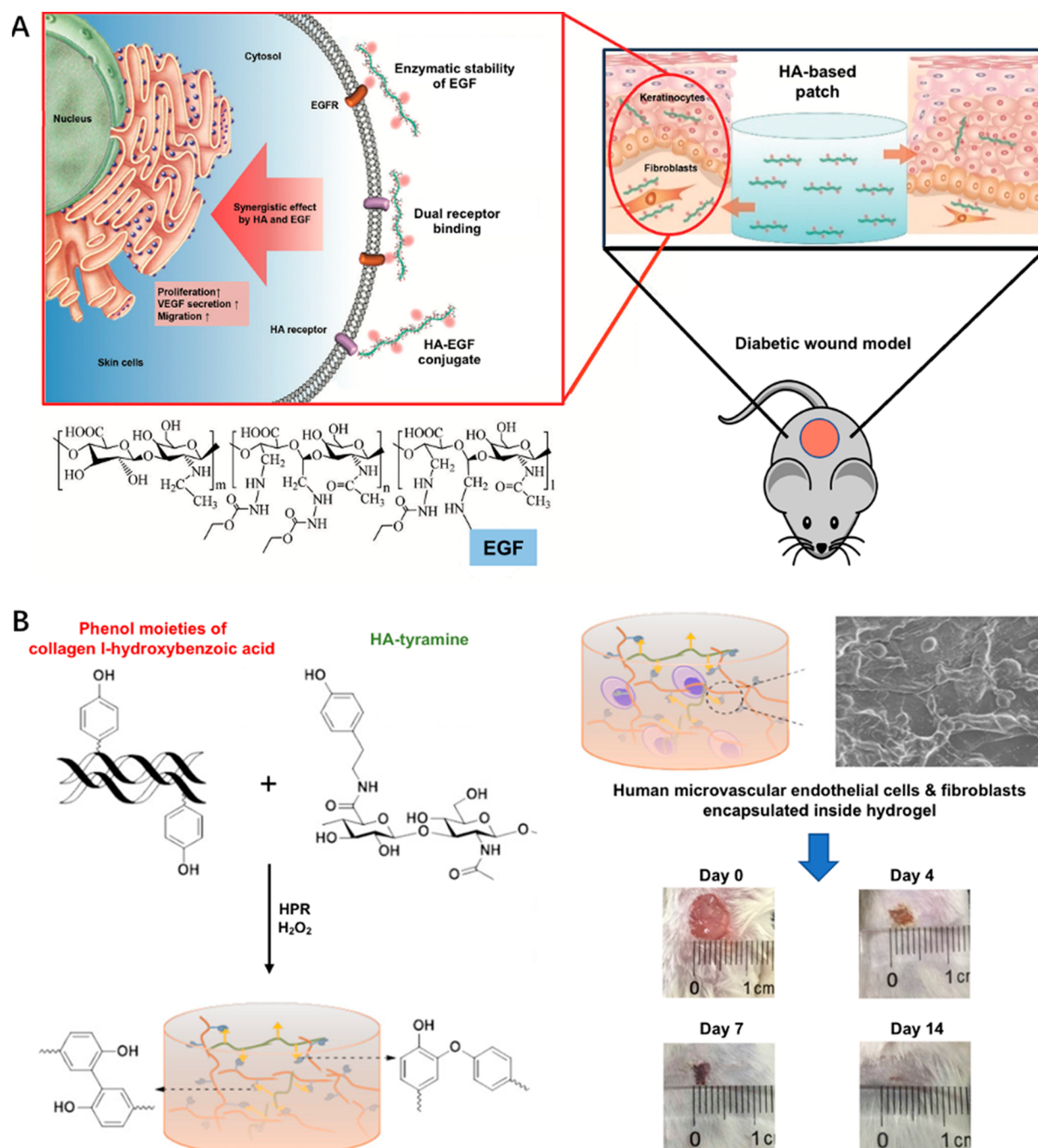


Figure 5. (A) HMW-HA-epidermal growth factor (EGF) conjugate patch. Patch provides synergistic effects for diabetic wound healing. Adapted with permission from ref ¹⁵¹. Copyright 2018 Royal Society of Chemistry. (B) *In situ* formed HMW-HA-collagen hydrogel promoting wound healing. Adapted with permission from ref ¹⁵⁰. Copyright 2019 Elsevier.

skin cells (e.g., keratinocytes and fibroblasts), achieving significantly improved therapeutic efficacy in excisional full-thickness wounds of diabetic rats as compared to the formulations containing either HA or EGF (Figure 5A). This healing outcome is likely due to the fact that the HMW-HA-EGF conjugate could enhance the accumulation of EGF on skin cells *via* HA-CD44 interaction.¹⁵¹

In addition, when HMW-HAs are cross-linked with other polymers, wound dressings with distinct structures, such as sponges,¹⁵⁵ films,¹⁵⁶ and hydrogels,¹⁵⁷ can be formed. Recently, a hydrogel was prepared by cross-linking HMW-HA-tyramine (200 kDa) with collagen I-hydroxybenzoic acid

under the stimulation of horseradish peroxidase and H₂O₂.¹⁵⁰ The hydrogel containing fibroblasts and human microvascular endothelial cells could significantly promote the proliferation of skin cells and the secretion of VEGF, resulting in effective healing in rats with excisional full-thickness wounds (Figure 5B).

4. CONCLUSION

HAs have been used for not only improving wellness (as cosmetics) but also treating skin diseases (as therapeutics). For example, a number of HA-based wound dressings have been developed for wound care, and several of them are currently

applied in clinic, which have been mentioned elsewhere.⁸¹ Recent developments of polymerization and functionalization methodologies facilitate the design of HA derivatives with control over the MW, degradation dynamics, and formulation (e.g., sponges, films, and hydrogels).¹⁵⁸ Emerging HA derivatives with multifaceted functions have provided therapeutic efficacy for treating serious damage and chronic wounds.^{159,160} Therefore, a combination regimen of HA derivatives and other therapeutic modalities (e.g., cytokine therapy and cell therapy)^{150,151} may potentially target all of the phases (hemostasis, inflammation, proliferation, and remodeling) throughout wound healing.

However, it must be borne in mind that the pathophysiological conditions underlying delayed healing are still not fully understood, and the ideal host(s) for the production of HAs are less effectively obtained. Although native HAs are generally considered biocompatible and biodegradable, they, particularly LMW-HAs, have the risk of allergic reactions; for example, the application of HAs may worsen skin ulcers in patients with dermatosclerosis (it is rare disease that involves the hardening and tightening of the connective tissues).¹⁶¹ In addition, chemical modification needs to be carefully adjusted to achieve appropriate substitution and/or cross-linking, generating HA derivatives with favorable properties on the basis of their specific demands.^{116,162} However, it is worth noting that HA derivatives with extensive chemical modifications may further cause unexpected toxicity and side effects. Furthermore, the large-scale production of hydrogels or scaffolds using HAs derivatives with the homogeneous and reproducible structure still presents a challenge. Therefore, HA-based regenerative biomaterials will only be widely applied when these aforementioned issues are fully addressed.

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H.Y. and J.G. discussed the need for the manuscript and wrote the original draft. L.S., D.S., Y.Z., L.W., and Z.Y. reviewed the draft with detailed comments. H.Y., L.S., D.S., Y.Z., L.W., Z.Y., and J.G. made the revisions. All authors read and approved the final manuscript for submission.

Notes

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