Universidade de Lisboa

Faculdade de Farmácia

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Hyaluronic acid (HA) and chitosan (CS) as wound dressing materials

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Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

Orientador: Doctor Teresa Cerchiara

Co-orientador: Professora associada com agregação, Doutora Helena Maria Cabral Marques

2020

Department of Pharmacy and Biotechnology

Università di Bologna

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Master of Science (MSc) in Pharmaceutical Sciences

2020

ABSTRACT

The skin is the largest organ of the human body, with the primary function of protection. When its normal anatomic structure and function is disrupted, a wound is formed. A phenomenon called wound healing is subsequently activated, normally divided into four phases, that occur in an overlapping manner. The first, hemostasis, aims at immediately protecting the injury site by blocking blood and fluid loss. Then, inflammatory agents are recruited, including neutrophils, lymphocytes, and macrophages, in the inflammatory phase. Proliferation comes next, where new granulation tissue is formed simultaneously with re-epithelialization, and angiogenesis, and, finally, tissue remodeling or scar formation occurs. This sequence of events, and the time in which they occur, determine the outcome of an acute or chronic wound. Chronic wounds often reoccur and are frequently associated with local or systemic factors, such as immunosuppression, chronic diseases, like diabetes, infection, and aging.

In the last decades, the high cost of wound management and increasingly aging population prone to the development of chronic wounds, have driven the research for more effective wound dressings. There is a wide variety of wound dressings currently available, with distinct characteristics, and a set of parameters should inform the better choice for each patient, and different types of wound. The novel wound dressings currently being studied, take into consideration the different phases of the wound healing process and the external factors that may impair it. Therefore, biopolymers such as hyaluronic acid and chitosan, have become popular as wound dressing materials, as they exhibit biocompatible and biodegradable properties, allied with a low cost and renewable nature. Hydrogels and films using these polymers, as well as delivery systems, namely microparticles, have been explored. This monograph aims at reviewing some of the recent advances in the field of wound dressings using hyaluronic acid and chitosan.

KEYWORDS: wound healing; hyaluronic acid; chitosan; hydrogels; films; microparticles.

RESUMO

A pele é o maior órgão do corpo humano, com função de proteção, regulação da temperatura corporal e suporte de vasos sanguíneos e nervos. Quando esta barreira é danificada, criando uma ferida, inicia-se um processo de cicatrização. Este é habitualmente dividido em quatro fases, que se sobrepõe. A primeira, a homeostase, visa o bloqueio imediato do sangramento da ferida, por mecanismos de vasoconstrição e coagulação. Subsequentemente, dá-se um processo inflamatório, com o recrutamento de neutrófilos, macrófagos e linfócitos para o local da agressão, seguindo-se uma fase de proliferação, com a formação de novo tecido de granulação, re-epitelização e angiogénese. Finalmente, ocorre a formação da cicatriz, na fase de remodelação. A sequência destes eventos determina a formação de uma ferida aguda ou crónica. As feridas agudas passam por todos os passos do processo no espaço de poucos dias, ou semanas, enquanto uma ferida crónica se prolonga no tempo, tendo muitas vezes fatores externos e internos associados. Entre estes, destacam-se a presença de infeção, doença crónica, como diabetes, imunossupressão e a idade.

O tratamento das feridas crónicas, e a sua manutenção, assume custos elevados para os sistemas de saúde, com perspetivas de aumento devido ao envelhecimento da população. Assim, a emergência de tratamentos mais eficazes tem sido uma área de grande exploração científica nas últimas décadas. Os novos tratamentos para feridas atualmente em estudo têm em consideração todos os passos da cicatrização, assim como os mecanismos envolvidos. Entre os materiais usados, destacam-se os biopolímeros, pela sua biocompatibilidade e biodegradabilidade, assim como o baixo custo e natureza renovável. Pelo seu papel na cicatrização das feridas, o ácido hialurónico e o quitosano são dois dos biopolímeros mais usados. Desse modo, esta monografia pretende explorar a utilização destes polímeros como material para tratamento de feridas, nas formulações atualmente em estudo.

PALAVRAS-CHAVE: ferida; cicatrização; ácido hialurónico: quitosano; hidrogel; film; micropartículas.

ACKNOWLEDGEMENTS

Terminados cinco anos de aprendizagem e crescimento pessoal, resta-me agradecer a todos aqueles que fizeram parte do meu trajeto, e a quem dedico o meu trabalho:

À minha família, em especial aos meus pais e ao meu irmão, pelo apoio incondicional, em todos os momentos. Pelas oportunidades que me proporcionam e por depositarem em mim a força e confiança que muitas vezes não tenho. Sem vocês, nada seria possível.

Aos meus amigos, os da faculdade, com quem as dúvidas e preocupações deram lugar a uma amizade incondicional que excedeu a FFUL. Ana, Afonso, Margarida, Joana, Francisca e Cândida, este percurso só faria sentido convosco, e há muito provaram que serão eternos.

À Inês, para quem anos de amizade tornam estas palavras redundantes, o apoio de sempre e para sempre.

To Professor Teresa Cerchiara, and all the members of the research laboratory in the University of Bologna, for welcoming me so generously. However short my Erasmus experience was, thank you for teaching me and for all the availability, even in the strangest of times.

Por último, à Professora Doutora Helena Marques, pela ajuda demonstrada ao longo do desenvolvimento desta monografia.

A todos, um sincero *obrigada*.

Abbreviations

- ADP - Adenosine diphosphate
- bFGF – basic Fibroblast growth factor
- CD44 – CD44 antigen
- CS - Chitosan
- DAMPs - Damage-associated molecular patterns
- DD – Deacetylation degree
- ECM - Extracellular matrix
- EGF – Epidermal growth factor
- ECs – Endothelial cells
- GAGs - Glycosaminoglycans
- GPCRs - G protein-coupled receptors
- HA – Hyaluronic acid
- HAS - HA synthases
- HYALs – Hyaluronidases
- HPCS - Hydroxypropyl chitosan
- ICAM-1 - Intercellular adhesion molecule
- IGF - Insulin growth factor
- IL-6 - Interleukin-6
- IL-1β - Interleukin-1β
- KGF - Keratinocyte growth factor
- M1 – Early-stage macrophage
- M2 – Activated macrophage
- MMPs – Metalloproteinases
- MW - Molecular weight
- NETs - Neutrophil extracellular traps
- PDGF - Platelet-derived growth factor
- ROS - Reactive oxygen species
- TGF-α - Transforming growth factor-α
- TGF-β - Transforming growth factor-β
- TNF – Tumor necrosis factor

- TSG-6 - TNF-stimulated gene-6
- VEGF - Vascular endothelial growth factor
- VCAM-1 - Vascular cell adhesion molecule
- vWF - von Willebrand factor

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Objectives and Method

The present monograph was initiated with the intent to study the development of a new formulation with Hyaluronic acid microcapsules containing Vitamin E, developed using the spray-drying technique, to incorporate in a film dressing for wound treatment (Annex A.1). Due to the impossibility of continuing the work in a laboratorial setting, a literature search was instead conducted to evaluate the current "state-of-the-art" in wound healing strategies that utilize biopolymers, such as Hyaluronic acid and chitosan.

Therefore, for the elaboration of this monograph, an extensive research of articles was carried out in the bibliographic databases *PubMed*, *ScienceDirect* and *ResearchGate*. The research was performed in English, between April and October of 2020, using the terms "Wound healing", "Wound dressings", "Hyaluronic acid", "Chitosan", "Hydrogel", "Wound film", "Microparticles", and "Wound drug delivery". The selection of the articles used was made based on their title and abstract. For the physiologic processes, pathophysiology, and description of biological structures no time limit was defined for research. As for the characterization of Hyaluronic acid and chitosan wound dressings, only the period of 2010-2020 was used to illustrate the most recent developments in the field.

1 INTRODUCTION

1.1 Skin

The skin constitutes the largest organ of the human body, which main function is the protection of internal organs and tissues, regulation of body temperature and supporting blood vessels and nerves (1,2). It is comprised of a stratified three-layer structure: epidermis, dermis, and hypodermis (**Figure 1**) (3).

The epidermis, the outermost layer, serves as a physical and chemical barrier between the interior body and exterior environment. It consists mainly of keratinocytes arrayed in four distinct substrata - the basal, spinous, granular and cornified layers - which synthesize the protein keratin, giving it its rigidity and permeability. Among the keratinocytes of the epidermis are intercalated melanocytes, which synthesize melanin and dispense it to numerous adjacent keratinocytes, Langerhans cells, responsible for immune response, and Merkel cells, associated with afferent nerve conduction and mechanoreception that is responsible for the light touch sensation (4). The epidermis has no blood vessels and is nourished by simple diffusion of nutrients from the underlying connective tissue (4).

The second layer, the dermis, is composed of dense collagenous connective tissue: it contains a vascularized extracellular matrix (ECM) rich in fibroblasts that produce type I and III collagen, reticulum fibers, elastin and glycosaminoglycans (GAGs) (4,5). It is responsible for the strength, elasticity, viscosity and hydration of the skin, supporting the vasculature, lymphatic system, and nerve bundles (5). The reticular layer in the dermis contains the hair follicles, sebaceous and eccrine glands, and Pacinian corpuscles, responsible for pressure detection (4,6). The most prevalent cell type in the dermis are fibroblasts, that possess an important purpose during the wound healing process, as they are responsible for the production of remodeling enzymes, such as proteases and collagenases.

Finally, the hypodermis, is constituted by adipose tissue and collagen (4). It contains larger blood vessels and nerves that are also found in the dermis. This skin layer acts as an insulator and conserves body heat.

When this structure is disrupted and a skin injury is formed, the cells within these three layers must coordinate at precise stages in order to promote healing (7), as will be described in the upcoming chapters.

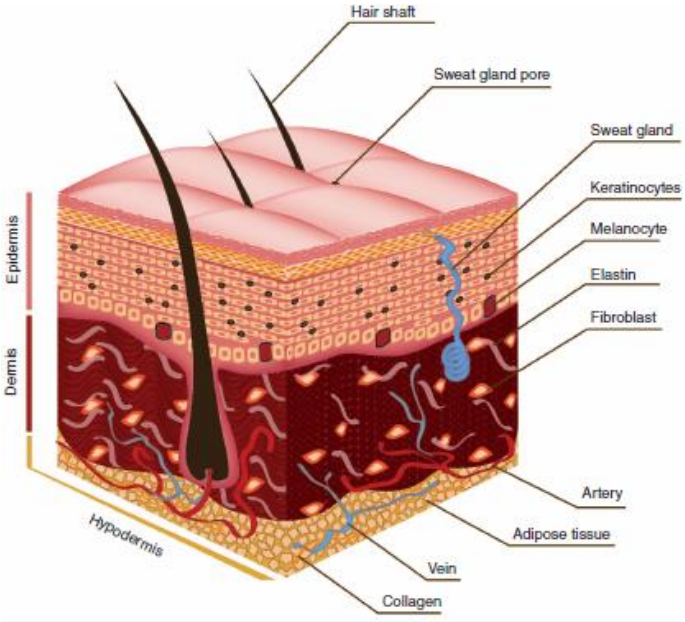

Figure 1 - Representation of the skin structure, highlighting the three layers, the skin appendages, and the main cellular constituents. The figure was taken from Pereira *et al*., 2013 (3).

1.2 Wounds

A wound is the result of the "disruption of normal anatomic structure and function" of the skin, according to the Wound Healing Society. To reestablish it, a wound healing process is activated, traditionally divided into four phases, depicted later in this monograph (section 1.2.2.). The normal occurrence of this phenomenon, or its failure, determines the classification of wounds as acute or chronic.

1.2.1 Acute and chronic wounds

As mentioned, the duration and nature of the healing process leads to the formation of an acute or chronic wound. Acute wounds are the result of sudden trauma and their healing is usually very efficient, passing through the different stages of the process in a matter of weeks,

depending on the size and number of layers of skin that have been affected (8–10). When the injury reaches complete re-epithelization, it no longer needs drainage or a dressing, since the connective tissue has been repaired (11).

When the reparation process fails, whether by its inability to reach a satisfactory anatomic or functional result, or by surpassing a period longer than 8-12 weeks to achieve healing, the wound is classified as chronic (12,13). Chronic wounds suggest a failure in some aspects of the repair process, often as early as in the inflammatory phase. This happens as a result of a continued recruitment of active neutrophils or an accumulation of apoptotic neutrophils (14,15). This type of wounds often reoccur and are frequently associated with poor primary treatment, persistent bacterial infections, repeated trauma and comorbidities, namely advanced age, peripheral vascular disease, malnutrition, diabetes, cancer and chronic steroid use (4,13). More details regarding the factors that affect wound healing will be given in section 1.2.3. of this monograph. Chronic wounds also exhibit higher concentrations of exudate, that contain proteinase enzymes, such as metalloproteinases (MMPs) and polymorphonuclear (PMN) elastase, that destroy the surrounding tissue, as well as an inadequate blood supply that leads to failure in the healing process (4,13,15). When studying therapeutic solutions for this type of wounds, it is important to consider all the underlying issues, as described by Strecker-McGraw *et al.* (4), summarized in **Table 1**.

It has been estimated that millions of people worldwide suffer with chronic wounds, an incapacitating disease that greatly disturbs the quality of life of those affected. Moreover, its treatment onus becomes a major health issue and a strain on resources, with the cost of supporting treatment to these patients representing hundreds of millions of dollars per year (16,17). The advanced wound care market in Europe has been estimated at USD 2.8 billion and this number is set to grow in the next few years, driven mainly by an increasingly aging population (18).

Table 1 - Clinical features present in chronic wounds. The table was adapted from Strecker-McGraw *et al*., 2007 (4).

CLINICAL FEATURES OF CHRONIC WOUNDS
• Absence of healthy granulation tissue • Presence of necrotic and unhealthy tissue at the injury site • Excess exudates • Lack of adequate blood supply • Failure of re-epithelialization • Cyclical or persistent pain • Recurrent disruption of the wound • Clinical or subclinical infection

1.2.2 Wound healing phases

The physiological process that restores tissue integrity after trauma is known as wound healing. This highly precise and coordinated process is traditionally broken down into four intersecting phases (**Figure 2**): hemostasis, inflammation, proliferation (synthesis and replication stages), and tissue remodeling or scar formation (9,19). Succinctly, hemostasis occurs to immediately seal the wound in order to prevent blood loss, followed by the inflammatory phase, that acts to prevent infection, giving place to the differentiation of mesenchymal cells, and their proliferation and migration to the healing site, appropriate angiogenesis, rapid re-epithelialization, meaning, the re-growth of the epithelium at the wound surface, and, finally, the reorganization of the ECM around the injury site, in the remodeling phase (20,21). The disturbance of one or more of these phases, as previously mentioned, could result in damaging outcomes, either creating a hypertrophic scar or contributing to the formation of a chronic wound (22).

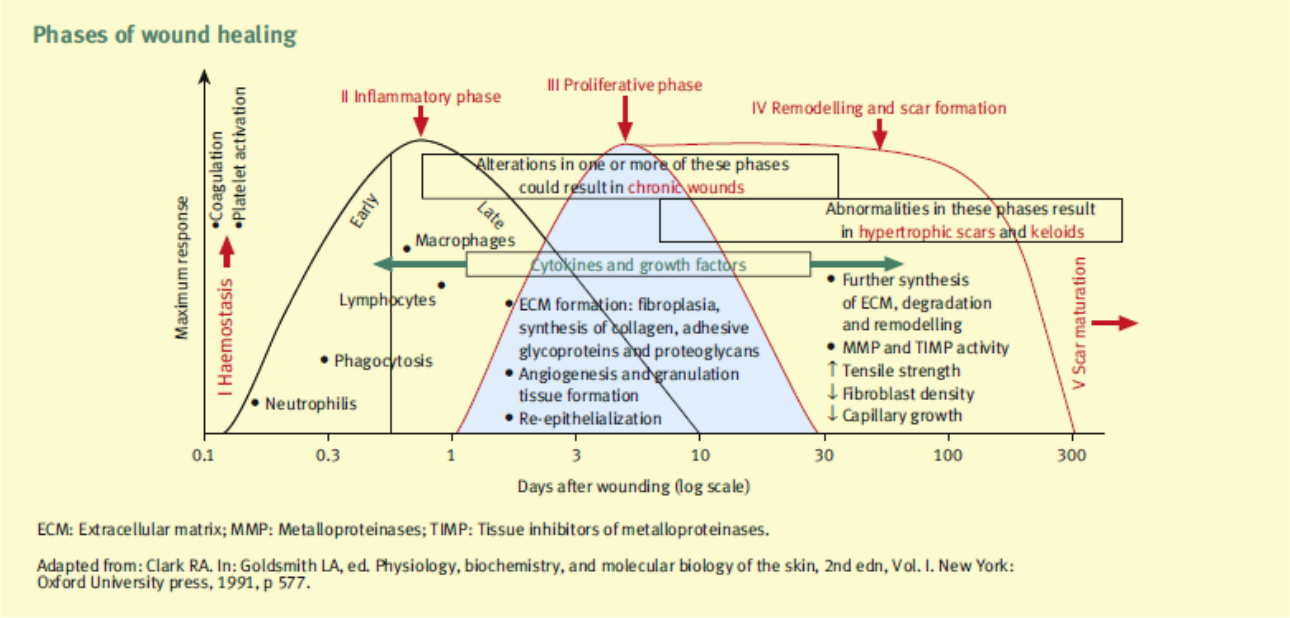

Figure 2 - Intersecting phases of wound healing. Depiction of the process through time, highlighting the main occurrences of each phase: I – Hemostasis; II – Inflammatory phase; III – Proliferative phase; IV – Remodeling and scar formation. The figure was taken from Enoch *et al.* 2008 (11).

1.2.2.1 Hemostasis

Hemostasis (**Figure 3 - A**) is the first step of wound healing, that occurs by exposure of the subendothelial wall of the vessels to blood components, acting immediately to prevent fluid and blood losses (7,23). In this process, it is possible to establish three different phases: vasoconstriction, primary hemostasis, and secondary hemostasis (7).

Vasoconstriction: After the tissue damage, the immediate response is vasoconstriction, triggered by vasoconstrictors released by the wounded endothelium – such as endothelin – and regulated by circulating catecholamines, epinephrine, norepinephrine, and prostaglandins released from injured cells (7,19). This response is executed by the reflexive contracture of the vascular smooth muscle, however, causes only temporary stoppage of bleeding (7). This happens because of passive relaxation of the muscle, caused by increasing hypoxia and acidosis of the wound. Then, in order to resolve bleeding, the activation of the coagulation cascade must happen (7).

Primary hemostasis: When injury occurs and the blood vessels rupture, the thrombogenic subendothelial matrix is exposed, causing the platelets to bind through receptors on their surface (7). Subsequentially, the inside-out signaling pathway is activated, instigating the attachment of platelets to other platelets, using G protein-coupled receptors (GPCRs), integrins, and glycoproteins on their surface, and the ECM – binding with collagen, fibrinogen, fibronectin, and the von Willebrand factor (vWF) (7). What follows is the activation of the inside-in signaling pathway, leading to a surge in platelet activation and modulates the actin cytoskeleton, altering the actin conformation (7). This, in turn, modifies the platelet shape, developing pseudopodia and lamellipodia, which strongly attaches to the ECM, contracts, and mechanically seals the blood vessel (7). The activated platelet secretes active substances such as adenosine diphosphate (ADP), serotonin, calcium, and histamine that are required for platelet activation as well as vWF and integrins, which will also be needed to secondary hemostasis (7). The platelet plug is therefore formed by binding vWF and collagen in the subendothelial matrix and the platelet aggregation caused by the release of thromboxane A_2 from activated platelets (7). Platelets provide the surface for the assembly and activation of coagulation complexes, but its absence does not impede the wound healing process (7).

Secondary hemostasis: The intrinsic and extrinsic coagulation pathways culminate in the activation of factor X, and thus prothrombin gets converted into thrombin, which cleaves fibrinogen into fibrin (7). Factor XIII covalently crosslinks fibrin, which binds the aggregated platelet plug forming a secondary hemostasis plug – the fibrin clot. The fibrin clot will serve as a provisional wound matrix for the infiltration of other cells in the following stages of wound healing (24). Moreover, as the platelets in the clot suffer degranulation, they release pro-inflammatory cytokines and growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), and insulin growth factor (IGF) that will prompt the next stages of healing: these mediators influence neutrophils, monocytes/macrophages, smooth muscle cells, endothelial cells, and fibroblasts, ultimately leading to the initiation of the inflammatory response (7,19,25).

1.2.2.2 Inflammatory phase

The inflammatory stage (**Figure 3 - B**) begins with the recruitment of inflammatory cells, such as neutrophils, to the wound site, followed, a few days later, by monocytes that later differentiate into macrophages, and lymphocytes (4,19,21,23).

Mast cells are known to recruit inflammatory cells immediately after injury, while releasing inflammatory cytokines, vasodilation agents, vascular permeability factors, and proteases that increase the recruitment of immune cells into the wound, (7).

Neutrophils are the first circulating inflammatory cells to be recruited to the site of the wound in response to substances released by cells in the injured area, such as calcium waves, damage-associated molecular patterns (DAMPs), hydrogen peroxide, lipid mediators, and chemokines (7,14,25). Neutrophils become the most prevalent cell in the wound a few days after injury, destroying infectious agents by releasing toxic granules, producing reactive oxygen species (ROS) that lead to an oxidative burst, initiating phagocytosis, and generating neutrophil extracellular traps (NETs), in which neutrophils extend chromatin filaments coated with proteases in order to eliminate pathogens (7,26). It is also important to note the significant role of these cells in the resolution of inflammation, as it is their elimination from the injured area that provides the signal for it to initiate (14). This can happen either by being engulfed by macrophages through efferocytosis or by re-entering the circulation through a process called reverse migration (7). Failure of this process leads to the emergence of chronic wounds, as mentioned earlier, as a result of an upsurge in neutrophils at the wound site (14).

Macrophages are critical to normal wound healing and tissue regeneration, and differentiate from monocytes, that are recruited within the first 24-48h of wound formation in response to chemokines and transcription factors responsive to hypoxia, in conjunction with platelet and mast cell degranulation (7,26). These cells will phagocytose pathogens and tissue debris, while also releasing a variety of growth factors, chemokines, and cytokines (20). Macrophages that appear in the early stages of wound healing, usually referred to as M1, are microbicidal and pro-inflammatory, expressing tumor necrosis factor (TNF-α), interleukins (IL)-6 and IL-1β (7). After inflammation, the M1 phenotype transitions into the M2 macrophage or alternatively activated macrophage (7). This type of macrophage is anti-inflammatory and releases growth factors, such as vascular endothelial growth factor (VEGF), the most prevalent angiogenic factor during skin healing, contributing to new vessel formation (4,7).

Lymphocytes infiltrate the wound later in the inflammatory phase, supporting fibroblast proliferation and collagen biosynthesis (25).

At the end of the inflammatory phase, there is deposition of a protein matrix, composed of fibronectin, collagen, and hyaluronic acid. This phase terminates with the engulfing of dying neutrophils by macrophages (7,13).

1.2.2.3 Proliferative phase

During the proliferative phase (**Figure 3 - C**) of wound healing the formation of new granulation tissue happens simultaneously with other healing processes, including re-epithelialization, angiogenesis, and the formation of granulation tissue (7). This processes are stimulated and modulated predominantly by (a) fibroblasts, that secret IGF-1, basic fibroblast growth factor (bFGF), TGF-β, PDGF, and EGF, (b) endothelial cells, responsible for the synthetization of VEGF, bFGF, and PDGF , and (c) keratinocytes, that synthesize TGF-α and TGF-β (25).

Granulation tissue formation: The granulation tissue is composed of macrophages, endothelial cells and, most importantly, fibroblasts, that help contract the wound and synthesize new ECM, while also serving as a scaffold for other cells and components – including, as formerly stated, new blood vessels and inflammatory cells (7,20). Olczyk *et al.* (25) describes that fibroblasts are attracted to the injured area according to the chemotactic PDGF, EGF, IGF, and TGF-β gradient, while growth factors stimulate its proliferation. The granulation tissue is created by collagen, in particular types I and III, elastin, proteoglycans, glycosaminoglycans, and non-collagenous proteins synthesized primarily by fibroblasts whose activity is regulated by PDGF and TGF-β, secreted mostly by platelets and macrophages (25). Moreover, PDGF also stimulates the expression of collagenase, while TGF-β regulates the accumulation of ECM components (25).

It is also worth mentioning the role of hyaluronic acid (HA) during this phase, existing in great amounts in the matrix of the early granulation tissue, along with fibronectin. Before its concentration starts to decrease giving place to collagen, the hyaluronic acid molecules create a woven structure that enables the coming cells to penetrate the wound area (25).

Later in the wound healing process, during remodeling, the granulation tissue is replaced by normal connective tissue (7).

Re-epithelialization: During this process, epithelial cells migrate to the injury site, proliferate, and differentiate, stimulated by growth factors, such as EGF, keratinocyte growth factor (KGF), and TGF-α, that are produced by activated wound macrophages, platelets and keratinocytes (27). The migration of these cells comes to a stop when they achieve a uniform layer.

Angiogenesis: In order for the wound healing process itself to happen, the formation of new blood vessels, a process denominated angiogenesis, is essential, allowing for the delivery of nutrients and maintenance of oxygen homeostasis, which enhances cellular proliferation and tissue regeneration (7). VEGF and PDGF, present in the hypoxic environment of the wound, activate local microvascular endothelial cells (ECs) – thus initiating angiogenesis (7,9). Other mediators, such as bFGF, TGF-β, TNF-α, angiogenin and angiotropin secreted by epithelial cells, fibroblasts and macrophages also aid this process (25). As described by Rodrigues *et al.* (7), the activated ECs break down ECM in the granulation tissue, proliferate, migrate, form new cell-cell junctions, and expand to form new capillaries (7). The interaction of the endothelial cells with leukocytes represents a critical factor in skin repair. This contact is made possible by glycoprotein receptors expressed by ECs in the microenvironment of the wound, of which are example P-selectin and E-selectin, that facilitate adhesion and infiltration of leukocytes into the skin. In addition, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 are upregulated by ECs, detaining the movement of leukocytes.

1.2.2.4 Tissue remodeling phase

Remodeling (**Figure 3 - D**), the last stage of healing, is also the longest – after wound closure, wounds can continue to undergo remodeling or tissue maturation for several months or even years (7). During this phase, the major event is wound contracture, led by the phenotypic differentiation of the fibroblasts existing in the wound into myofibroblasts. Additionally, integrin receptors $\alpha_1 \beta_1$ and $\alpha_2 \beta_1$ mediate the contraction of the granulation tissue by interacting with collagen (25). The granulation tissue matures, evolving to scar tissue.

At this stage, all the processes that were initiated after wounding are gradually terminated. The majority of the endothelial cells, macrophages and myofibroblasts suffer apoptosis and the type III collagen deposited during the proliferative phase is slowly degraded and replaced with type

I collagen, effectively changing the composition of the ECM, resulting in formation of scar tissue (13,20,23).

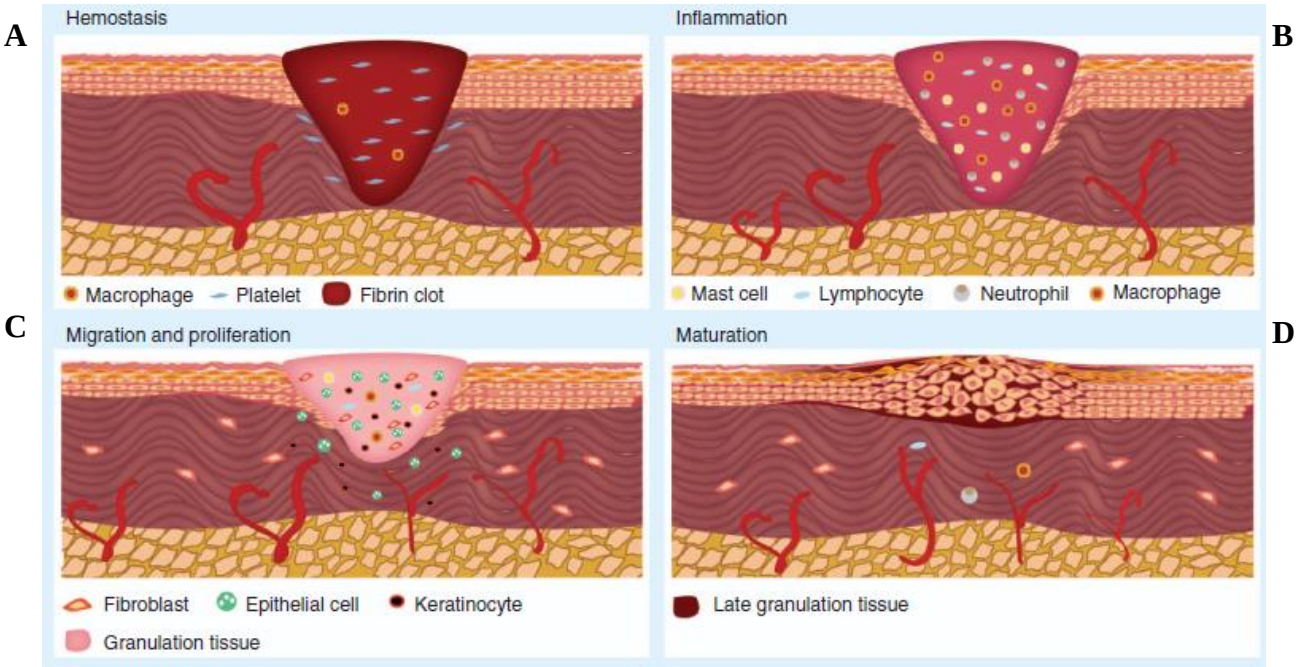

Figure 3 – Schematic representation of the four wound healing phases: (A) Hemostasis; (B) Inflammation; (C) Migration and proliferation; and (D) maturation. The figure was taken from Pereira *et al*. 2013 (3).

1.2.3 Factors affecting wound healing

As mentioned earlier, there are multiple factors, usually classified as local or systemic, that can affect wound healing, leading to chronic wound formation (28). Such factors can hinder one or more phases in the process, compromising tissue repair (28). Local factors, namely oxygenation, infection, and venous sufficiency, directly affect the characteristic of the wound itself. As for systemic factors, they affect the overall health or disease state of the individual, therefore compromising the ability to heal (28). Both are generally interconnected.

According to Singh *et al.* in a 2017 review (9), some factors to consider are nutrition, hypoxia, infection, immunosuppression, chronic disease, wound management, and age, amongst others:

a) Nutrition – Some nutrients are critical for wound healing, including vitamin A (involved in epidermal growth), carbohydrates (for collagen synthesis) and omega-3 fatty acids

(modulates the arachidonic acid pathway) (9). Therefore, a deficient nutrition can affect healing by prolonging inflammation, inhibiting fibroblast function, and reducing angiogenesis and collagen deposition (9).

b) Hypoxia – For the wound healing process to occur in an adequate manner, a balanced level of oxygenation must exist: while a degree of hypoxia is required to attract neutrophiles and macrophages and facilitate re-epithelialization, sufficient oxygen is an essential requirement for phagocytosis and collagen deposition (9).
c) Infection – It is crucial for the healing process that the wound bed stays clean and free of contamination. Before surgery, antibiotic prophylaxis reduces the risk of infection (9).
d) Immunosuppression – Diseases like cancer or HIV impair the inflammatory response and can impede the healing cascade (9). Also, persisting medication with oral steroids can decrease cytokine concentrations during wound repair, leading to reduced collagen deposition (9).
e) Chronic disease – Diabetes is well known to impair wound healing, affecting leukocyte function due to high levels of glucose, and alter MMP expression and function (9). Additionally, it causes microvascular damage, affecting tissue oxygen levels and the supply of nutrients (9).
f) Wound management – Wound dressings for the treatments of skin injuries should follow a certain set of characteristics, that will be discussed in the next section of this monograph.
g) Age – Not only do elderly patients have more likelihood to suffer from chronic diseases that, as already mentioned, impair wound healing, they also have a thinner epidermal layer and slower inflammatory, migratory and proliferation responses (9).

2 WOUND DRESSINGS

In order to successfully treat and manage wounds, it is compulsory to understand the underlying mechanism of wound formation, the type of wound, the healing process and the general condition of a patient when it comes to its health, as seen in previous sections of this monograph. Therefore, a proper treatment can be chosen. There are many wound dressings currently available and its classification varies, as will be described later in this chapter. For the healing to be properly conducted, the wound dressing should verify a certain set of parameters and many developments have been made in previous years to create dressings that closely achieve them.

2.1 Characteristics of an ideal wound dressing

The ideal wound dressing should try to replicate the characteristics of the skin to achieve adequate healing results. Because of the distinct characteristics of the different types of wounds and of each of the wound healing stages, there is not a unique dressing that can be efficiently applied in all situations (29). Even so, the ideal wound dressing should observe a certain set of characteristics: (a) capacity to provide optimal moisture wound environment, while also avoiding the accumulation of wound exudates; (b) ability to maintain appropriate local temperature in order to ensure proper tissue perfusion and, therefore, enhance epidermal migration; (c) biocompatibility; (d) biodegradability; (e) semi-permeability to water and oxygen; (f) hypoallergenic properties, guaranteeing that no allergic or immune response happens; (g) compatibility with topical therapeutic agents, being able to act as drug delivery systems, incorporating and releasing drugs to the wound bed; (h) be non-adherent to the wound and easy to remove without causing trauma; (i) and lastly, be cost effective (**Table 3**) (6,10,30).

As described by Sood *et al.* (31), to create and maintain a moist, clean and warm environment, where wound healing is most likely to thrive, four basic principles should be involved when choosing an optimal dressing: (a) “if a wound appears to be dry or desiccated, appropriate hydration must be provided” (31); (b) “if a wound produces excessive exudates, the fluid needs to be absorbed” (31); (c) “if a wound has necrotic tissue or evident debris, it will need debridement” (31) (d) and, finally, “if a wound is infected, it needs to be treated with the appropriate antibacterial agent” (31). All these principles should be taken into consideration, while also pushing toward the achievement of the ideal characteristics mentioned above.

Although a wound dressing that meets all the requirements is difficult to attain, it is possible to optimize the physical and chemical properties of wound dressing materials, as to meet most of the wound needs at a particular wound stage.

2.2 Wound dressing classifications

Wound dressings can be classified according to different parameters, depending on the type of material used for healing, function in the wound, physical form or ability to provide a moist environment to the wound (3,32). Furthermore, they are divided into traditional or modern dressings, and active ingredients, such as antimicrobials and growth factors, could be incorporated into medicated dressings which will be useful in wound healing either directly or indirectly (32).

The traditional treatment of wounds intends primarily to keep the wound dry by allowing evaporation of wound exudate and preventing the entry of harmful bacteria into the wound, using natural or synthetic bandages such as cotton or gauzes (32). This type of dressings has a very high absorption capacity; however, they can cause bleeding and damage of newly formed epithelium upon removal from the wound surface. In addition, exudate leaking from traditional dressing materials usually increases the risk of infection and the material can cause foreign body reaction, being some of the most significant problems of these type of dressings (6).

It is currently known that dressings that guarantee a moist environment are the most suitable for fast re-epithelialization (30). That said, the advancements to develop new, more modern, wound dressings have been in the direction of more comprehensive systems that take into consideration the different phases of wound healing and the opportunities to act in each one, as well as in the factors affecting healing. Polymers have become popular as wound dressing materials, having exhibited *in vitro* and *in vivo* wound healing properties, as well as the capacity to incorporate microparticulate and nanoparticulate delivery systems (32). Based on their nature of action, wound dressings are classified into three main groups: inert/passive, bioactive, and interactive (1). This classification, as described by Negut *et al.* (1) is presented in **Table 2**.

Table 2 - Wound dressing classification. The table was adapted from Negut *et al.* 2018 (1).

TYPE OF DRESSING	FORMULATION	ADVANTAGES	DISADVANTAGES
Inert/passive	Gauzes	Have great porosity and thermal isolation. Are available in multiple forms, including bandages, sponges, and plasters, and can be applied directly to the surface of suppurating wounds.	Are mostly suitable for minor wounds and highly absorbent, which may cause dryness. Therefore, they can stick to wounds, and disrupt the wound bed when removed.
Bioactive	Hydrocolloids	Are semi-permeable in the form of solid wafers and can enclose hydroactive particles that swell with exudates or form a gel. Moreover, are useful to low or moderate exudating wounds and can be detached from the injury site without difficulty, as well as being considered painless dressings.	Can be cytotoxic, can possess an unpleasant odor, and present a low mechanically strength.
	Alginates	Are highly absorbent, therefore applicable for moderate to heavy exudating wounds, as well as bleeding wounds. Are helpful in debridement of shedding wounds.	Have limited use on low exudating wounds, causing dryness and scabbing, and should be changed daily.
	Collagens	Exist in the form of pads, gels, or particles and encourage the formation and setting of newly formed collagen in wounds. Furthermore, they can absorb heavy exudates, while offering a moist environment to wounds. They are easy to apply, non-immunogenic, and non-pyrogenic.	Not recommended for application in wounds with necrosis and third-degree burns; require a secondary dressing.

	Hydrofibers	Are soft nonwoven pads or ribbon dressings that absorb exudates and provide a moist environment in a deep wound, while offering a reduced risk of skin maceration.	Have limited absorption capacity, with excess causing an undesirable swelling of the dressing, leading to distension and possible loss of adhesion.
Interactive	Hydrogels	Rehydrates dry wounds, are easy to remove and change, have a high capacity to accumulate and absorb large volumes of water inside their polymeric network. Are permeable to metabolites, non-irritant, and non-reactive with biological tissues.	May cause over-hydration, weak mechanical properties, consequently necessitating a secondary dressing.
	Semi-permeable films	Have high transparency, allowing for wound check, and are highly elastic, permitting flexibility and adjusting to the form of the wound; are waterproof and permeable to oxygen. Can be used as an additional layer for hydrogels and foams.	Are mostly suitable for superficial wounds with limited exudate and for wound epithelialization.
	Semi-permeable foams	Can absorb large amounts of exudates and offer thermal isolation.	Can cause dryness and scabbing when applied to low exudating wounds and dry scars.

Table 3 - Characteristics of an ideal wound dressing. The table was taken from Sood *et al.* 2014 (31).

CHARACTERISTICS OF AN IDEAL WOUND DRESSING
• Creates a moist, clean, and warm environment. • Provides hydration if dry or desiccated. • Removes excess exudates. • Prevents desiccation and is nontraumatic. • Provides protection to periwound area. • Allows for gaseous exchange. • Impermeable to microorganisms. • Free of toxic or irritant particles. • Does not release particles or fibers. • Can conform to wound shape. • Minimal pain during application and removal • Easy to use. • Cost-effective.

3 BIOPOLYMERS AS WOUND HEALING MATERIALS

As mentioned previously, historically, the wound dressing's purpose was to shield the injured area from external contamination and mechanical stress. With recent developments in the field, wound dressings can have an active function in the healing process, functionalized with different therapeutic complexes delivered to the injury site, providing a sustained action (1). Some of these new delivery systems are based on the use of biomaterials, specifically biopolymers, and are especially relevant in the management of chronic wounds, as they eliminate the need for frequent administration of drugs, such as antibiotics and anti-inflammatory drugs and bioactive agents such as growth factors, in conventional dosage forms (16,33). This creates clear advantages, increasing patient compliance, enhancing the safety of the drug administration, and improving the stability of susceptible drugs (16).

The use of biopolymers for pharmaceutical applications has been a matter of interest in recent years, mainly due to its diverse composition, adjustable physical behavior, and wide variety, as well as its low cost of and renewable nature (34). Its applications as wound care materials are reinforced by their characteristics of biocompatibility, ability to support cell growth, regenerative potential, biodegradability, and durability (**Figure 4**) (29,35). Furthermore, the biopolymer-based wound dressing takes advantage of the capacity of these polymers to absorb large volumes of water when in the dry state and donate water when hydrated; their ability to integrate drugs, allowing for controlled release directly into the target site; and its usage as scaffolds for cell delivery and tissue engineering (34).

Next, three examples of novel drug delivery systems for wound healing –hydrogels, films, and microparticles - will be discussed, with emphasis on the use of biopolymers. Then, the wound healing potential of hyaluronic acid (HA) and chitosan (CS), both involved in the cascade of events leading to wound closure, will be highlighted.

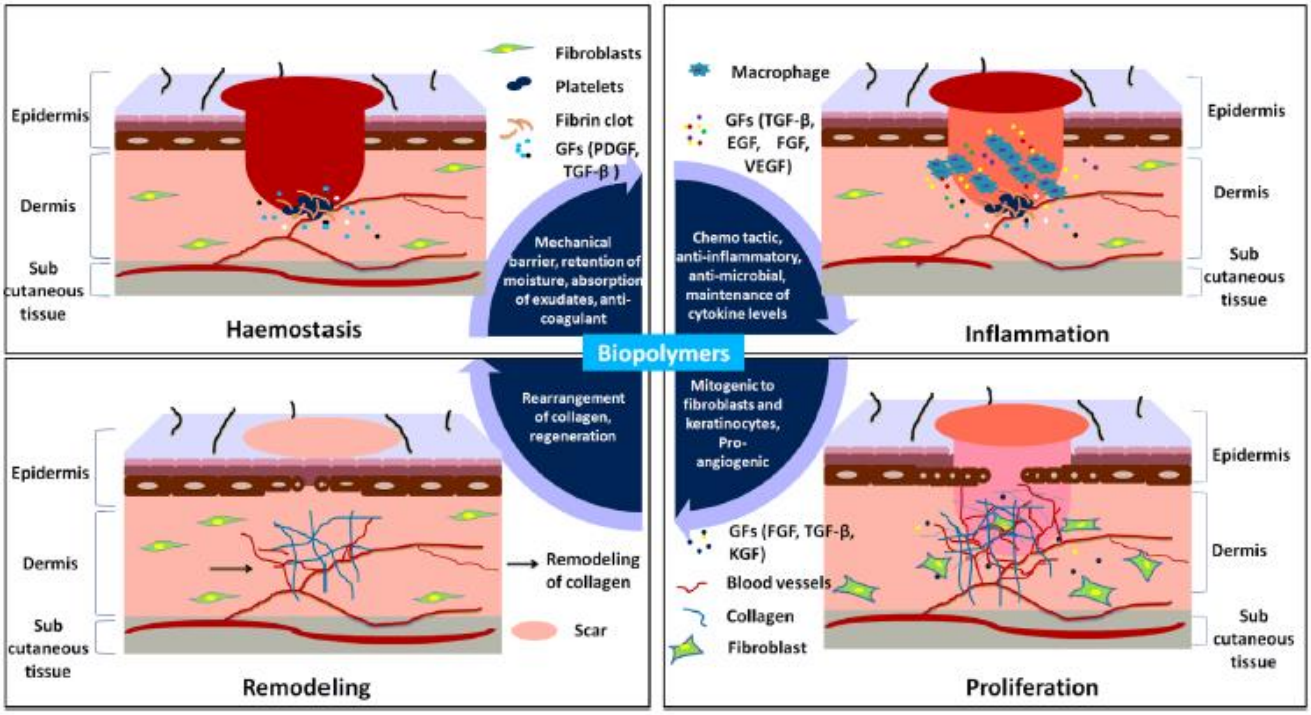

Figure 4 - Phases of wound healing and their correlation with the role of biopolymers. The figure was taken from Sahana *et al.* 2018 (35).

3.1 Hydrogels

Rodríguez-Rodríguez *et al.* (36) described hydrogels as "three-dimensional, hydrophilic, crosslinked polymeric networks able to swell and hold large amounts of water or biological fluids without losing their structure" (36). By definition, as a gel, it should contain a liquid phase, exceeding 90% of the volume, and a solid phase, that assures its consistency, making it able to absorb large quantities of water while remaining insoluble in the liquid phase (37). Its similarity to living tissues, biocompatibility and biodegradability make them suitable for a wide range of biomedical applications, in particular for tissue engineering, drug delivery systems and wound dressings (36).

Hydrogel dressings are the most suitable wound healing agent, possessing most of the desirable characteristics of an ideal wound dressing, described previously. They are capable of incorporating liquids, such as water or wound exudates, considerably lowering the risk of dressing-dependent infections (30). Furthermore, they are soft and bendable, which minimizes the damage to the surrounding tissue during and after application (37). Additionally, because

hydrogels are designed to hold moisture at the wound surface, they maintain skin hydration, allowing the enhancement of autolytic debridement with the removal of necrotic tissue, while also retaining the wound exudates, which, in turn, promotes fibroblast proliferation and keratinocyte migration, necessary for complete epithelialization and healing of the wound (29,32,38). Moreover, they are easy to prepare and allow sustained release of drugs, that can be entrapped into hydrogel networks during gelling process (38,39). However, they are more efficient in the treatment of wounds with less exudates because of its large quantity of water, thus being more appropriate to use for light to moderately exuding wounds (29). Their application in wounds with excessive exudate can originate wound maceration and lead to healing problems.

Different types of materials have been employed as wound dressings, such as natural polymers (30). The next sections of this monograph will focus on the application of two of these natural polymers as hydrogels: HA and CS.

3.2 Films

Films are thin membranes, normally durable, conformable, adhesive and easy to manipulate, permitting the exchange of oxygen and water vapor between the wound bed and the environment while remaining impermeable to liquid and bacterial contaminants (40). This type of wound dressing is not absorbent, and should only be used in wounds with few exudates (40).

3.3 Microparticles

The sustained release of a therapeutic agent to the injury site is one of the motivator factors in the most recent research developments in the wound dressing area. There are many ways of achieving it, notoriously using liposomes, microparticles or nanoparticles (41). Microparticles vary in size from 1µm to 1000µm and drug encapsulation is generally accomplished with polymeric, waxy, or other protective materials, that are synthetic, natural or modified natural polymers (41). Natural polymers are commonly used for the preparation of microparticulate systems for skin wound healing and drug delivery (41).

Degim *et al.* (41) explains that the most commonly selected polymers in wound healing are biodegradable and biocompatible polymers because of their surface properties, that include hydrophilicity, permeability and degradability, and their biocompatibility with tissues and blood (41). Additionally, Kim *et al.* (42) describes that "microparticle-mediated delivery would offer a more sustained therapeutic effect if only extracellular delivery is required because the lower surface-to-volume ratio would slow the release kinetics" (42). Moreover, the drugs are released from the microparticle through discharge from the polymer or by degradation of the polymer matrix (43).

Drug loaded polymeric microspheres are usually prepared by oil-in-water emulsification which yields a product with a broad size distribution (44).

An advantage of microparticles when compared with nanoparticles is that they act locally, possibly toxic substances can be carried encapsulated, and liquids can be handled as solids in the form of dried microparticles (45). Microparticles can be classified as microspheres or microcapsules: microspheres are matrix systems where the drug is homogeneously dispersed, either dissolved or homogenously suspended; microcapsules, however, are heterogenous particles where a membrane shell is surrounding the core forming a reservoir (45).

4 HYALURONIC ACID AND CHITOSAN-BASED WOUND DRESSINGS

4.1 Hyaluronic acid

4.1.1 Structure and properties

To properly understand the function of HA in wound healing, a revision of its structure and properties is necessary. HA is a linear and non-sulfated GAG, most often referred to as hyaluronan, due to the fact that it exists *in vivo* as a polyanion and not in the protonated acid form (46,47). It consists of a repeating disaccharide β-1,4-glucuronic acid and β-1,3 *N*-acetylglucosamine bound with β-glycosidic linkages (**Figure 5**) (13,33).

HA can be found in multiple animal tissues, with different concentrations and molecular weights, including vitreous humor, articular cartilage, synovial fluid, and the epidermis and dermis of the skin (48).

There are three enzymes responsible for the production of HA at the inner surface of the plasma membrane – the so-called HA synthases (HAS1, HAS2, HAS3) (49,50). HAS3 synthetizes HA with lower molecular weight (1×10^5 to 1×10^6 Da) than HAS1 and HAS2 (2×10^5 to 2×10^6 Da) (50). The needless of exocytosis allows HA to be readily able to interact with other cells and HA-binding proteins, the hyaladherins (49). Furthermore, HA catabolism happens in two primary ways: (a) through six hyaluronidases (HYALs), responsible for adjusting the various HA sizes required during each wound healing phase, or (b) via ROS.

As will be described next, HA is an important molecule within the ECM and assumes a particularly relevant role in various phases of wound healing (13).

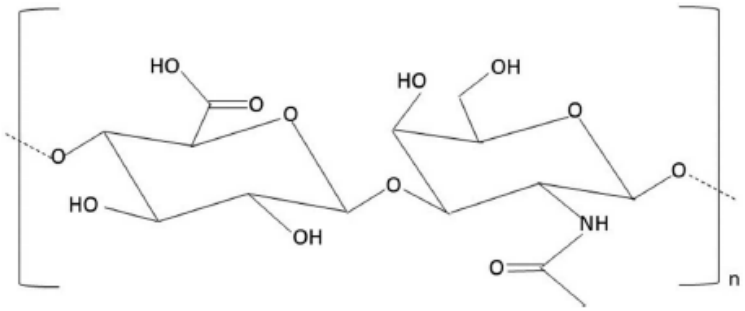

Figure 5 - Chemical structure of Hyaluronic acid. The figure was taken from Vigani *et al.* 2019 (13).

4.1.2 Applications in wound healing

HA significant role in wound healing has already been described in literature (51). Its functions consist of stimulating fibroblast proliferation, remodeling of ECM and keratinocyte migration (35). Therefore, these characteristics in combination with its biocompatibility, biodegradability, non-immunogenicity, and viscoelasticity, make a good argument for its use in a wide range of wound dressings (22,46).

To further comprehend the various functions of HA already mentioned, is necessary to make a distinction between high molecular weight HA and low molecular weight HA, as they are determined by its polymer molecular weight (MW). High MW hyaluronan, present at the first stages of wound healing, has anti-inflammatory, antiangiogenic and immunosuppressive properties, while also presenting a structural function (47). Low MW hyaluronan, on the other hand, has pro-inflammatory activities and is involved in later stages of the wound healing process, stimulating fibroblast proliferation and collagen synthesis during the remodeling phase (35,47). These processes will now be explained in more detail.

HA in the inflammatory phase of wound healing: During this phase, HA fragments are synthetized from platelets or dislocated to the site of injury from the blood stream, binding to fibrinogen and thus initiating the extrinsic coagulation pathway (49). The hydrophilic properties of HA lead to edema of the tissue surrounding the wound, opening spaces for infiltration of granulocytes for removal of tissue debris and bacteria (49). On the other hand, HA acts reducing and moderating the inflammatory response, through its contact with the hyaladherin TNF-stimulated gene-6 (TSG-6), which, in turn, is stimulated by IL-1 and TNF-α (49). This will

result in the expression of TSG-6 protein by inflammatory cells, amongst them the fibroblasts, that will bind with high MW HA, forming a heavy chain that effectively prevents inflammation by blocking neutrophil migration (49).

HA in the proliferative phase of wound healing: Small HA fragments attract fibroblasts to the area of injury, at a rate that is not only affected by HA MW but also by its molar concentration – as mentioned above, low MW HA is effective in stimulating proliferation of these cells (49). So, indirectly, HA influences the production of collagen, synthetized by fibroblasts. This collagen, along with elastin, form the fibrous scaffolding of the ECM, and high MW HA acts as a "cushioning gel", filling in the gaps and providing structural organization to the newly formed granulation tissue, as described by Frenkel *et al.* (49). Moreover, the degradation product of HA has a pro-angiogenic effect, binding to the hyaladherin CD44, stimulating MMPs (35,49). Finally, HA acts in the epithelization, facilitating the differentiation of keratinocytes by binding to CD44 receptors thus activating a series of cascades (35). CD44 acts as the major cell-surface hyaluronan receptor (13,50).

HA in the remodeling phase of wound healing: HA Contributes to scar formation, whether in acute or chronic wounds (49).

For these reasons, HA and HA-based materials have been extensively used in wound dressings, such as hydrogels, with *in vitro* and *in vivo* tests showing positive results for wound regeneration, as will be described in the next sections.

4.1.2.1 Hyaluronic acid hydrogels

HA is a material of interest due to its biocompatibility, biodegradability, and promotion of cell growth, amongst others, as mentioned before. Nevertheless, HA still presents some shortcomings, which include poor mechanical properties and rapid degradation *in vivo* (52). These problems can be overcome through modification of the (a) carboxyl groups, to form amide bonds, (b) hydroxyl groups, to ether formation, ester formation, hemiacetal formation, and oxidation and (c) -$NHCOCH_3$, by deacetylation, amidation, hemiacetylation, and hemiacetal formation by using chemical modification or crosslinking, improving mechanical

properties, degradation, viscosity, solubility, and biologic properties, as described by Chircov *et al.* (52).

In a 2017 study, Shi *et al.* (53) used dynamic metal–ligand coordination bonds to fabricate moldable supramolecular HA hydrogels with self-healing properties. To achieve reversible crosslinking of HA chains, the biopolymer was modified with pendant bisphosphonate (BP) ligands using carbodiimide coupling and chemo-selective "click" reactions (53). *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) were used to evaluate the bacterial inhibition activities of HA-BP · Ag^+, demonstrating inhibition to both, lead mostly by the action of $AgNO_3$, since HA alone was not effective against these strains (53). Therefore, HA can be used in combination with broad-spectrum antibacterial agents (53). Moreover, HA-BP·Ag^+, showed significant skin regeneration capacities and wound healing abilities, promoting regeneration of the epidermal layer and angiogenesis at the wound site (53). These results demonstrated that this HA-based hydrogel could be an excellent wound healing material.

Hong *et al.* (54) studied two kinds of hyaluronic acid (HA)-based hydrogels: one made from physical freezing-thawing of HA solution, and the other from chemical cross-linking of HA and polysaccharide. They were applied in the repair of full-thickness skin defects with New Zealand rabbits as the test animals, using powder HA and cotton dressings as the references (54). In comparison to traditional dressings, both the hydrogels demonstrated better biocompatibility. Nevertheless, the chemical cross-linked HA hydrogel showed the most promising results, reducing the scar formation via reduction of TGF-β1 levels, and improving VEGF expression, promoting skin regeneration (54).

4.1.2.2 Hyaluronic acid-based microparticles

Babo *et al.* (55) developed hyaluronic acid microparticles for the controlled delivery of growth factors using a spray/dehydration method (55). The method used by Babo *et al.* consists of the "injection of a solution of HA and adipic acid dihydrazide (ADH) through a nozzle to produce a beam of particles that are collected in a solution containing carbodiimide, which promotes the crosslinking of the particles by a reaction previously described for the production of HA hydrogels and microparticles" (55). This reaction occurs at room temperature, being an

improvement when compared to the high temperatures employed in the solvent evaporation and spray-drying methods, risking thermally denaturing thermosensible compounds (55). Therefore, this method allowed the usage of native HA. Growth factors were then incorporated, aiming to extend their activity (55). These HA microparticles demonstrated stability and potential to deliver growth factors, what could have important implication in the treatment of non-healing wounds (55).

4.2 Chitosan

4.2.1 Structure and properties

CS is composed of randomly distributed glucosamine and N-acetylglucosamine units linked through β(1–4) glycosidic bonds (**Figure 6**) (13). It is the deacetylated derivative of poly-*N*-acetyl-d-glucosamine, also known as chitin (**Figure 7**) – the second most abundant biopolymer in nature after cellulose (13,33). Chitin is found in the exoskeletons of crustaceans, mollusks, and insects, as well as in the cell walls of fungi and bacteria (13). The deacetylation that transforms chitin in CS occurs under alkaline conditions and is, in addition to its molecular weight, a requirement for its functionality in a wide variety of applications (13). Depending on the source and preparation, CS's deacetylation degree (DD) varies between 60% and 100% and its molecular weight ranges from 300 kDA to 1000 kDA (37). Moreover, its solubility is affected by pH: in acidic pH, CS is a cationic polyelectrolyte, soluble in aqueous media, since the amino groups are protonated and, consequently, positively charged; on the contrary, in neutral and basic solutions, the amino groups are deprotonated and CS becomes insoluble (13,56). A summary of the relation between structural characteristics and properties of chitosan can be found in **Table 4**.

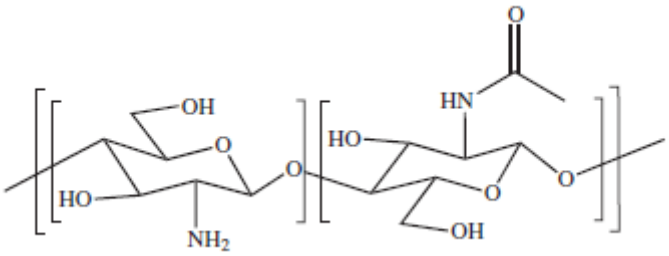

Figure 6 - Chemical structure of chitosan. Figure taken from Croisier *et al.* 2012 (37).

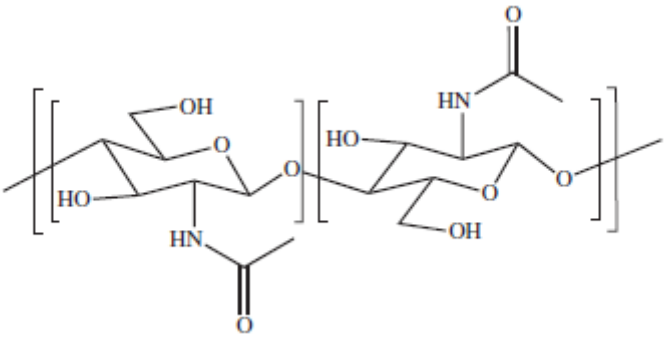

Figure 7 - Chemical structure of chitin. Figure taken from Croisier *et al.* 2012 (37).

Table 4 - Relation between structural characteristics and properties of chitosan. The table was taken from Dash *et al.* 2011 (56).

Property	Structural characteristics[a]
Solubility	↑ DD
Crystallinity	↓ DD
Biodegradability	↓ DD, ↓ Molecular weight
Viscosity	↑ DD
Biocompatibility	↑ DD
Biological	
Mucoadhesion	↑ DD, ↑ Molecular weight
Analgesic	↑ DD
Antimicrobial	↑ DD, Molecular weight
Permeation enhancing effect	↑ DD
Antioxidant	↑ DD, ↓ Molecular weight
Hemostatic	↑ DD

[a] ↑ – Directly propotional to property; ↓ – inversely propotional to property.

4.2.2 Applications in wound healing

Chitin and CS are the most applied biomaterials for wound healing, due to being easily processed into gels, membranes, nanofibers, microparticles, nanoparticles, scaffolds and sponge-like forms (15,57). CS exhibits an unique property amongst other biodegradable polymers – its cationic character, given to it by its primary amino groups, as well as film-forming and gelation characteristics that are responsible for many features that makes it attractive to use in drug delivery systems, including for the treatment of chronic skin ulcers, comprising hydrogels, sponge-like dressings, and films (13,41,58). The properties of chitosan that make it an attractive biomaterial to use in wound dressing are (a) its high biocompatibility, (b) excellent rate of biodegradability, (c) resistance against environmental conditions, (d) effective antifungal and antimicrobial activity against both gram-positive and gram-negative strains, (e) non-toxicity, (f) adhesive nature, (g) excellent oxygen permeability, (h) tissue adhesive properties and (i) its effective role as a healing enhancer across all stages of wound healing (33,38,57).

CS has been proven to stimulate hemostasis, while its properties are also notorious in the inflammation stage, when CS activates PMN migration and macrophage phagocytosis, thus removing foreign agents from the wound and stimulating ECM deposition and re-epithelization

(13,59). CS also has cell proliferative activities, by activating polymorphonuclear leucocytes and macrophages for phagocytosis and production of IL-1, TGF-β, and PDGF, and also stimulating fibroblast proliferation and activation (35). Moreover, as mentioned previously, the exudate of chronic wounds has singular properties, including the over-expression of MMPs, that can weaken the remodeling phase of the wound healing process by exercising a corrosive effect on ECM components (13). CS acts inhibiting the activation of these enzymes, successfully preventing the negative outcome of its actions. If a hypertrophic scar if formed, by excessive collagen production in the remodeling phase, CS can decrease scar tissue, allowing for a good re-epithelialization. Additionally, CS affects the expression of growth factors implied during the healing process (59).

4.2.2.1 Chitosan hydrogels

In an acidic environment, CS allow the formation of gels. CS-based hydrogels are of great interest, since they are biocompatible and present activity against pathogenic microorganisms (22). Okur *et al.* (22) described the main limitations of CS-based hydrogels as their weak anti-bacterial performance and unsatisfactory mechanical properties, instigating research on the use of CS in combination with other polymers to further improve its hydrophilic nature, increase antibacterial effect and enhance mechanical properties (22).

Lu *et al.* (60), used photocross-linkable hydroxypropyl CS (HPCS) and 4-azidobenzoic hydroxypropyl chitosan (Az-HPCS) to form in-situ cross-linked bioadhesive hydrogel membrane and evaluate its potential as a novel wound dressing (60). The results showed that the hydrogel membrane possessed a dense and stable structure in a hydrated state, and was capable of removing exudate from wounds, while maintaining a hydrated environment (60). Moreover, bacteria penetration was studied, revealing that the hydrogel was able to act as a protective barrier, whilst allowing for the penetration of oxygen. Finally, *in vitro* studies using dermal fibroblast and epidermal keratinocytes showed no cytotoxicity caused by the hydrogel membrane, nor effects on cell proliferation (60). It was then possible to conclude that the in-situ photocross-linked azidobenzoic hydroxypropyl CS hydrogel membrane has interest characteristics for the treatment of wounds (60).

Another study by Mozalewska *et al.* (61) with the intent to develop an antimicrobial hydrogel wound dressing constituted by lower weight CS in lactic acid, using radiation-initiated

crosslinking of hydrophilic polymers, showed that the molecular weight and degree of deacetylation influence solubility of CS: higher molecular weight of CS leads to a decrease in solubility (61). Preliminary studies on the antibacterial activity of CS-containing hydrogel dressings showed CS suppresses the growth of Gram-positive bacteria to greater extent than Gram-negative bacteria (61). This study reassured the value of hydrogel dressings in the context of wound healing.

4.2.2.2 Chitosan films

CS derivative films are relevant especially because of their high antimicrobial activities (22).

A study conducted by Gunes *et al.* (62) aimed at developing *Hypericum perforatum* oil incorporated CS films (62). It was shown that the prepared CS based films would provide an adequate level of moisture without any wound dehydration, as well as antimicrobial activity against Gram-negative bacteria, *E.coli*, and Gram-positive bacteria, *S.aureus* (62). Additionally, these films were able to provide an adequate level of moisture to the wound area, and high swelling properties, which could be used in moderate to highly exuding wounds (62). The films showed biocompatibility, proving they are non-cytotoxic and can provide a good matrix for cell proliferation as indicated on fibroblast cells (62). The combination of the antibacterial effect and wound healing properties indicates that chitosan films with H. *perforatum* oil could be an innovative and novel bioactive dressing material (62).

4.2.2.3 Chitosan-based microparticles

CS-based microparticles can be prepared by reacting CS with controlled amounts of multivalent anions resulting in cross-linking between the CS molecules (21). Then, the drug release from CS microparticles can be controlled by cross-linking the matrix using chemical cross-linking agents or by ionic cross-linking (21).

Romic *et al.* (63) attempted to develop melatonin-loaded CS-based microspheres as dry powder formulation suitable for wound dressing, using the spray-drying method (63). The results showed that entrapment of melatonin in CS/Pluronic® F127 microspheres potentiated CS antimicrobial activity against *Staphylococcus aureus* and five clinical isolated *S. aureus* MRSA

strains (63). This wound dressing was able to rapidly absorb exudate in the wound and provide an optimal water vapor transmission rate (63). Therefore, it has great potential at not only removing excessive wound exudation, but also treat bacterial infections in the injured area (63).

5 CONCLUSIONS

For an effective wound management, it is necessary to know the process of wound healing and its determinants, as well as the conditions that may affect it. In recent years, various novel wound dressings have been studied in order to improve wound care and respond to the need to reduce the cost of treatments for health systems while addressing the necessity of proper care of an increasingly aging population suffering with chronic wounds.

Among the newest developments, biopolymers have emerged as effective wound dressing materials, due to its biocompatibility, biodegradability, and non-cytotoxicity. Many of these polymers have been used effectively, thus, chitosan and hyaluronic acid can be recognized among them. Their association with drugs aiming at resolving infection in the wound, is one example of a positive outcome of the newly explored dressings, showing successful results *in vitro* and *in vivo* both with hyaluronic acid-based dressings, as well as chitosan-based dressings. Despite the studies already conducted and the great potential demonstrated, there are still few options in the market. In the near future, more exploration should be made in that sense.

REFERENCES

1. Negut I, Grumezescu V, Grumezescu AM. Treatment Strategies for Infected Wounds. Molecules. 2018;23(9):1–23.
2. Gary D. Hammer SJM. Pathophysiology of Disease : An Introduction to Clinical Medicine. Seventh. Gary D. Hammer SJM, editor. McGraw-Hill Education; 2014. 779 p.
3. Pereira RF, Barrias CC, Granja PL, Bartolo PJ. Advanced biofabrication strategies for skin regeneration and repair - Review. Nanomedicine. 2013;8(4):603–21.
4. Strecker-McGraw MK, Jones TR, Baer DG. Soft Tissue Wounds and Principles of Healing. Emergency Medicine Clinics of North America. 2007;25(1):1–22.
5. Zhong SP, Zhang YZ, Lim CT. Tissue scaffolds for skin wound healing and dermal reconstruction. WIREs Nanomedicine and Nanobiotechnology. 2010;2(5):510–25.
6. Sezer AD, Cevher E. Biopolymers as Wound Healing Materials: Challenges and New Strategies. Biomaterials Applications for Nanomedicine, Rosario Pignatello, IntechOpen. 2011;
7. Rodrigues M, Kosaric N, Bonham CA, Gurtner GC. Wound healing: A cellular perspective. Physiological Reviews. 2019;99(1):665–706.
8. Simões D, Miguel SP, Ribeiro MP, Coutinho P. Recent advances on antimicrobial wound dressing: A review. European Journal of Pharmaceutics and Biopharmaceutics. 2018;127:130–41.
9. Singh S, Young A, McNaught C-E. The physiology of wound healing. Surgery (Oxford). 2017;35(9):473–7.
10. Dhivya S, Padma VV, Santhini E. Wound dressings – a review. BioMedicine. 2015;5(4):24–8.
11. Enoch S, Leaper DJ. Basic science of wound healing. Surgery (Oxford). 2008;26(2):31–7.
12. Järbrink K, Ni G, Sönnergren H, Schmidtchen A, Pang C, Bajpai R, et al. Prevalence and incidence of chronic wounds and related complications: a protocol for a systematic review. Systematic Reviews. 2016;5:1–6.

13. Vigani B, Rossi S, Sandri G, Bonferoni MC, Caramella CM, Ferrari F. Hyaluronic acid and chitosan-based nanosystems : a new dressing generation for wound care wound care. Expert Opinion on Drug Delivery. 2019;16(7):715–40.

14. Wilgus TA, Roy S, McDaniel JC. Neutrophils and Wound Repair: Positive Actions and Negative Reactions. Advances in Wound Care. 2013;2(7):379–88.

15. Zarei F, Soleimaninejad M. Role of growth factors and biomaterials in wound healing. Artificial Cells, Nanomedicine, and Biotechnology. 2018;0(0):1–6.

16. Pachuau L. Recent developments in novel drug delivery systems for wound healing. Expert Opinion on Drug Delivery. 2015;12(12):1895–909.

17. Tricco AC, Cogo E, Isaranuwatchai W, Khan PA, Sanmugalingham G, Antony J, et al. A systematic review of cost-effectiveness analyses of complex wound interventions reveals optimal treatments for specific wound types. BMC Medicine. 2015;13(90):1–16.

18. Global Advanced Wound Care Market Size, Share, Trends, Growth Analysis Report - Segmented By Type (Dressings, Therapy Devices & Active Wound Care), By Application (Skin Ulcers, Surgical Wounds & Burn Wounds), By End-User (inpatient Services & Outpatient S [Internet]. Market Data Forecast. 2020 [cited 2020 Oct 16]. p. 175. Available from: https://www.marketdataforecast.com/market-reports/advanced-wound-care-market

19. Thiruvoth F, Mohapatra D, Sivakumar D, Chittoria R, Nandhagopal V. Current concepts in the physiology of adult wound healing. Plastic and Aesthetic Research. 2015;2(5):250–6.

20. Profyris C, Tziotzios C, Vale I Do. Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics. Journal of the American Academy of Dermatology. 2012;66(1):1–10.

21. Ranjan S, Fontana F, Ullah H, Hirvonen J, Santos HA. Microparticles to enhance delivery of drugs and growth factors into wound sites. Therapeutic Delivery. 2016;7(10):711–32.

22. Okur ME, Karantas ID, Senygit Z, Okur NÜ, Siafaka PI. Recent trends on wound management: New therapeutic choices based on polymeric carriers. Asian Journal of Pharmaceutical Sciences. 2020;

23. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration.

Nature. 2008;453(7193):314–21.

24. Martin P. Wound Healing — Aiming for Perfect Skin Regeneration. Science. 1997;276(5309):75–81.

25. Olczyk P, Mencner L, Komosinska-Vassev K. The Role of the Extracellular Matrix Components in Cutaneous Wound Healing. BioMed Research International. 2014;

26. Eming SA, Krieg T, Davidson JM. Inflammation in Wound Repair: Molecular and Cellular Mechanisms. Journal of Investigative Dermatology. 2007;127(3):514–25.

27. Tsala DE, Dawe A, Habtemariam S. Natural wound healing and bioactive natural products. Phytopharmacology. 2013;4(3):532–60.

28. Guo S, DiPietro LA. Factors Affecting Wound Healing. Critical Reviews in Oral Biology & Medicine. 2010;89(3):219–29.

29. Boateng JS, Matthews KH, Stevens HNE, Eccleston GM. Wound Healing Dressings and Drug Delivery Systems: A Review. Journal of Pharmaceutical Sciences. 2008;97(8):2892–923.

30. Mihai MM, Dima MB, Dima B, Holban AM. Nanomaterials for Wound Healing and Infection Control. Materials. 2019;12(13):1–16.

31. Sood A, Granick MS, Tomaselli NL. Wound Dressings and Comparative Effectiveness Data. Advances in Wound Care. 2014;3(8):511–29.

32. Ghadi R, Jain A, Khan W, Domb AJ. Microparticulate polymers and hydrogels for wound healing. Vol. 2, Wound Healing Biomaterials - Volume 2. Elsevier Ltd.; 2016. 203–225 p.

33. Kumar SSD, Rajendran NK, Houreld NN, Abrahamse H. Recent advances on silver nanoparticle and biopolymer based biomaterials for wound healing applications. International Journal of Biological Macromolecules. 2018;115.

34. Smith AM, Moxon S, Morris GA. Biopolymers as wound healing materials. Vol. 2, Wound Healing Biomaterials - Volume 2. Elsevier Ltd; 2016. 261–287 p.

35. Sahana TG, Rekha PD. Biopolymers : Applications in wound healing and skin tissue engineering. Molecular Biology Reports. 2018;

36. Rodríguez-rodríguez R, Espinosa-andrews H, Velasquillo-Martínez C, García-carvajal ZY. Composite hydrogels based on gelatin, chitosan and polyvinyl alcohol to biomedical

applications: a review. International Journal of Polymeric Materials and Polymeric Biomaterials. 2019;1–20.

37. Croisier F, Jérôme C. Chitosan-based biomaterials for tissue engineering. European Polymer Journal. 2013;49(4):780–92.

38. Kamoun EA, Kenawy E-RS, Chen X. A review on polymeric hydrogel membranes for wound dressing applications: PVA-based hydrogel dressings. Journal of Advanced Research. 2017;8(3):217–33.

39. Bhattacharya D, Ghosh B, Mukhopadhyay M. Development of nanotechnology for advancement and application in wound healing: a review. IET Nanobiotechnology. 2019;13(8):778–85.

40. Öztürk F, Ermertcan AT. Wound healing: a new approach to the topical wound care. Cutaneous and Ocular Toxicology. 2011;30(2):92–9.

41. Zelihagul D. Use of microparticulate systems to accelerate skin wound healing. Journal of Drug Tergeting. 2008;16(6):437–48.

42. Kim HS, Sun X, Lee J-H, Kim H, Fu X, Leong KW. Advanced drug delivery systems and artificial skin grafts for skin wound healing. Advanced Drug Delivery Reviews. 2019;146:209–39.

43. Freiberg S, Zhu XX. Polymer microspheres for controlled drug release. International Journal of Pharmaceutics. 2004;282:1–18.

44. Chen W, Palazzo A, Hennink WE, Kok RJ. The effect of particle size on drug loading and release kinetics of gefitinib-loaded PLGA microspheres. Molecular Pharmaceutics. 2016;

45. Lengyel M, Kallai-Szabo N, Antal V, Laki AJ, Antal I. Microparticles, Microspheres, and Microcapsules for Advanced Drug Delivery. Scientia Pharmaceutica. 2019;87(3).

46. Liao Y, Jones SA, Forbes B, Martin GP, Brown MB. Hyaluronan: Pharmaceutical Characterization and Drug Delivery. Drug Delivery. 2005;12(6):327–42.

47. Litwiniuk M, Krejner A, Grzela T. Hyaluronic Acid in Inflammation and Tissue Regeneration. Wounds. 2016;28(3):78–88.

48. Chen LH, Xue JF, Zheng ZY, Shuhaidi M, Thu HE, Hussain Z. Hyaluronic acid, an efficient biomacromolecule for treatment of inflammatory skin and joint diseases: A

review of recent developments and critical appraisal of preclinical and clinical investigations. International Journal of Biological Macromolecules. 2018;116:572–84.

49. Frenkel JS. The role of hyaluronan in wound healing. International Wound Journal. 2012;

50. Jiang D, Liang J, Noble PW. Hyaluronan in Tissue Injury and Repair. Annual Review of Cell and Developmental Biology. 2007;23:435–61.

51. Neuman MG, Nanau RM, Oruña-Sanchez L, Coto G. Hyaluronic Acid and Wound Healing. Journal of Pharmacy and Pharmaceutical Sciences. 2015;18(1):53–60.

52. Chircov C, Grumezescu AM, Bejenaru LE. Hyaluronic acid-based scaffolds for tissue engineering. Romanian Journal of Morphology & Embryology. 2018;59(1):71–6.

53. Shi L, Zhao Y, Xie Q, Fan C, Hilborn J, Dai J, et al. Moldable Hyaluronan Hydrogel Enabled by Dynamic Metal – Bisphosphonate Coordination Chemistry for Wound Healing. Advanced Healthcare Materials. 2017;7(5):1–9.

54. Hong L, Shen M, Fang J, Wang Y, Bao Z, Bu S, et al. Hyaluronic acid (HA)-based hydrogels for full-thickness wound repairing and skin regeneration. Journal of Materials Science: Materials in Medicine. 2018;

55. Babo PS, Reis RL, Gomes ME. Production and characterization of hyaluronic acid microparticles for the controlled delivery of growth factors using a spray/dehydration method. Journal of Biomaterials Applications. 2016;0(0):1–15.

56. Dash M, Chiellini F, Ottenbrite RM, Chiellini E. Chitosan — A versatile semi-synthetic polymer in biomedical applications. Progress in Polymer Science. 2011;36(8):981–1014.

57. Ahmed S, Ahmad M, Jayachandran M, Qureshi MA, Ikram S. Immunochemistry & Chitosan Based Dressings for Wound Care. Immunochemistry & Immunopathology. 2015;1(2):1–6.

58. Bernkop-schnürch A, Dünnhaupt S. Chitosan-based drug delivery systems. European Journal of Pharmaceutics and Biopharmaceutics. 2012;

59. Patrulea V, Ostafe V, Borchard G, Jordan O. Chitosan as a starting material for wound healing applications. European Journal of Pharmaceutics and Biopharmaceutics. 2015;97(November):417–26.

60. Lu G, Ling K, Zhao P, Xu Z, Deng C, Zheng H. A novel in situ-formed hydrogel wound

dressing by the photocross-linking of a chitosan derivative. Wound Repair and Regeneration. 2010;18:70–9.

61. Mozalewska W, R. Czechowska-Biskup, Olejnik AK, Wach RA, Ulanski P. Chitosan-containing hydrogel wound dressings prepared by radiation technique. Radiation Physics and Chemistry. 2017;

62. Güneş S, Tıhmınlıoğlu F. Hypericum perforatum Incorporated Chitosan Films as Potential Bioactive Wound Dressing Material. International Journal of Biological Macromolecules. 2017;

63. Romić MD, Klarić MŠ, Lovrić J, Pepić I, Cetina-čižmek B, Filipović-grčić J, et al. Melatonin-loaded chitosan/Pluronic® F127 microspheres as in situ forming hydrogel: An innovative antimicrobial wound dressing. European Journal of Pharmaceutics and Biopharmaceutics. 2016;

ANNEX

A.1. PREPARATION OF THE VITAMIN E ACETATE MICROCAPSULES

The o/w emulsion was prepared using the English method. The formulation is as follows:

EMULSION	
Arabic gum	2.5 g
Vitamin E acetate	5 g
Water	5 mL
Triglycerides	1.5 g
Aqueous polymer solution (HA)	83 mL

Microcapsules are posteriorly obtained using spray-drying technique, using the following experimental conditions:

- T inlet 160°C;
- T outlet 106°C;
- aspiration 100%;
- pump 25%;
- flux 600-700 L/h.

Mini Büchi spray dryer B-191 (Büchi Labortechnik AG, Flawil, Switzerland).

The next steps would be yield determination, efficiency of encapsulation and drug loading.