Universidade de Lisboa

Faculdade de Farmácia

Technological Advances in Cutaneous Wound Repair

Andreia Filipa Nobre Barroso

Mestrado Integrado em Ciências Farmacêuticas

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

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Resumo

A pele atua como a primeira linha de proteção contra o ambiente externo e fornece funções essenciais. Quando a sua integridade é comprometida devido a complicações mecânicas, físicas ou relacionadas com o metabolismo, é geralmente observado um maior risco de danos adicionais. Além disso, vários fatores levam ao comprometimento do processo de cicatrização, o que pode levar a feridas crónicas e, consequentemente, causar sofrimento, aumentar a suscetibilidade a infeções e diminuir a qualidade de vida dos doentes.

A presente tese de mestrado descreve brevemente a anatomia e fisiologia da pele, as mudanças estruturais e funcionais desta ao longo do tempo, a patogénese das feridas crónicas e as abordagens terapêuticas desenvolvidas para tratar infeções e estimular o processo de cicatrização. Assim, nesta tese de mestrado, focámo-nos em terapias mais tradicionais (como por exemplo, enxertos e pensos) e em abordagens inovadoras, das quais fazem parte os substitutos cutâneos e sistemas nanotecnológicos. Outros tipos de estratégias avançadas no tratamento de feridas incluem terapias com células estaminais e fatores de crescimento, terapia génica, terapia por pressão negativa, oxigenoterapia hiperbárica e outras.

De forma geral, esta dissertação cobre os recentes avanços tecnológicos nas duas últimas décadas e discute possíveis perspetivas no tratamento de feridas.

Palavras-chave: Feridas crónicas; Cicatrização; Pensos; Substitutos cutâneos; Nanotecnologia.

Abstract

The skin acts as the first line of protection against environmental exposure and provides essential functions. When its integrity is compromised due to mechanical, physical or metabolism-related issues, a higher risk of further damage is commonly observed. Besides, several factors lead to impaired wound healing, which may lead to chronic wounds and consequently cause suffering, increase the susceptibility of infections, and decrease the quality of life of patients.

The present master thesis briefly describes the anatomy and physiology of the skin, the structural and functional changes over time, chronic wound pathogenesis and therapeutic approaches designed to treat infections and stimulate the healing process. Hence, in this master thesis, we focused on both traditional therapies (e.g. grafts and wound dressings) and innovative approaches, including engineered skin substitutes and nano-based drug delivery systems. Other types of advanced wound care strategies include stem cells therapies, growth factors therapies, gene therapy vectors, negative pressure wound therapy, hyperbaric oxygen therapy and others.

This overview covers the latest advancements in the past two decades and discusses possible prospects in wound management.

Keywords: Chronic wounds; Wound healing; Wound dressings; Skin tissue engineering; Nanotechnology.

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List of abbreviations

aFGF/FGF-1	Acid fibroblast growth factor
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ASCs	Adipose-derived stem cells
α-SMA	Alpha-smooth muscle actin
Arg	Arginine
BC	Bacterial celulose
bFGF/FGF-2	Basic fibroblast growth factor
BM-MSCs	Bone marrow derived mesenchymal stem cells
CERs	Ceramides
CNPs	Cerium dioxide nanoparticles
CXCL2	Chemokine (C-X-C motif) ligand 2
Cur	Curcumin
CDs	Cyclodextrins
DNA	Deoxyribonucleic acid
DFU	Diabetic foot ulcer
DDSs	Drug delivery systems
EGF	Epidermal growth factor
EO	Essential oil
ECM	Extracellular matrix
GO	Graphene oxide
GM-CSF/G-CSF	Granulocyte-macrophage colony-stimulating factor
GFs	Growth factors
Glucosyl-CER	Glucosylceramide
HB-EGF	Heparin-binding EGF-like growth factor
HBOT	Hyperbaric oxygen therapy
HA	Hyaluronic acid
HIF1α	Hypoxia-inducible factor 1-alpha
IGF-1	Insulin-like growth factor 1
IFN-α	Interferon alpha
IFN-β	Interferon beta
IL	Interleukin
K	Keratin
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
MIC	Minimum inhibitory concentration
MCP-1/CCL2	Monocyte chemoattractant protein-1
NPs	Nanoparticles

NLCs	Nanostructured lipid carriers
NMF	Natural moisturizing factor
NPWT	Negative pressure wound therapy
PDGF	Platelet-derived growth factor
PRP	Platelet-rich plasma
PCL	Poly(ε-caprolactone)
PEG	Polyethylene glycol
PLCL	Poly(L-lactic acid-*co*-ε-caprolactone)
PLGA	Poly(lactide-co-glycolide)
PU	Polyurethane
PVA	Poly(vinyl alcohol)
PVP	Polyvinylpyrrolidone
PGE2	Prostaglandin E2
ROS	Reactive oxygen species
SSD	Silver sulfadiazine
SLNs	Solid lipid nanoparticles
SM	Sphingomyelin
SC	*Stratum corneum*
SG	*Stratum granulosum*
THNRs	Terbium hydroxide nanorods
TIMPs	Tissue inhibitors of metalloproteinases
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TGs	Transglutaminases
TNF-α	Tumor necrosis growth factor alpha
UV	Ultraviolet
UC-MSCs	Umbilical cord tissue-derived mesenchymal stromal cells
VEGF	Vascular endothelial growth factor

1. The integumentary system

The skin acts as the first line of protection against environmental exposure and provides essential functions, such as thermoregulation, sensory functions, hydration, immunological surveillance, homeostasis of fluids, electrolytes, and proteins, and synthesis of vitamin D (1,2). Skin integrity is an important factor in the maintenance of an effective barrier. However, internal and external factors contribute simultaneously to a progressive loss of skin integrity. These include gender, age, concurrent diseases such as diabetes, malnutrition, infection or an immunocompromised condition, smoking and alcoholism, environmental insults (mainly ultraviolet exposure), corticosteroids, antineoplastic agents, and radiotherapy. Wounds in the skin can either be classed as epidermal or deep, and in this regard it is important to identify the structural components of skin. (3,4).

1.1 Epidermis

The epidermis refers to the surface layer of the skin and is made of several morphological distinct layers, as illustrated in Figure 1: *stratum basale* or *stratum germinativum, stratum spinosum* or prickly layer, *stratum granulosum* (SG), and *stratum corneum* (SC) or horny layer (5). Only present on soles of feet and palms of hands, an additional layer can be identified between the SG and SC - *stratum lucidum* (6).

The SC is the outermost layer of the skin and functions to form a barrier against penetration by irritants and pathogen (5). The SC is composed of corneocytes and inter-corneocyte lipids, and both derived from keratinocytes as they migrate from the basal layer to the cornified layer changing from proliferating keratinocytes to mitotically inactive cells. This unique form of non-apoptotic programmed cell death is called cornification (or keratinization). In normal conditions, the proliferation rate in the basal layer is balanced by the process of cell shedding from the surface of the cornified layer- desquamation – and both are required for the maintenance of epidermal homeostasis (7). The keratinocytes journey takes approximately fourteen days and is responsible for the constant renewal of the skin (8).

The basal cell layer is situated above the dermal-epidermal junction separating the epidermis from the dermis and is composed of a single layer of cuboidal cells attached to the basement membrane (BM) through two types of integrin-dependent junctions: hemidesmosomes, which connect intermediate filaments such keratin to basal lamina, and focal adhesions, which attach the actin filaments to fibers of fibronectin (6,9,10). These

adhesions must be disrupted during the repair process, allowing cell detachment and subsequent migration of keratinocytes. In the basal layer, the keratinocytes in a proliferative state characterized by an infrastructure composed of keratin (K) intermediate filaments, K5 and K14, move upward to the spinous layer changing their shape from cuboidal to polyhedral (9). The cells in *stratum spinosum* are attached to their neighboring cells through desmosomes, structures composed primarily of glycoproteins, providing cellular adhesion and resistance to mechanical forces (11). The desmosomal attachments between the cells look like 'prickles' or 'spines', hence the name prickly layer and *stratum spinosum* (6). As keratinocytes ascend, intermediate filaments containing K1 and K10 replace those containing K5 and K14 found in basal layers. Keratin gives skin protective qualities against mechanical and non-mechanical stresses and structurally supports the regulation of apoptosis and protein synthesis (8).

In the SG, the keratinocytes become more flattened and lose their nucleus and organelles via cytoplasmic lysozymes (12). They express filaggrin, K2 and loricrin, which aggregate to form the typical cytoplasmic electron-dense keratohyalin granules (13,14). Later in the SC, filaggrin is digested by a variety of proteases, including caspase 14, into the amino acids components of natural moisturizing factor (NMF). NMF is mainly composed of amino acids such as urocanic acid, inorganic ions (e.g., potassium, sodium, calcium), pyrrolidone carboxylic acid and lactate, which collectively contribute to the maintenance of proper hydration, flexibility and possibly UV protection (11,15,16).

SC consists of corneocytes, flattened dead cells, interconnected by corneodesmosomes, modified desmosomal structures whose proteolysis influences the process of desquamation (17–19). Each corneocyte is enclosed by a specialized membrane, the cornified envelope, composed of keratins that are surrounded by an insoluble combination of proteins: loricrin (the main component), involucrin, filaggrin, trichohyalin and the class of small proline-rich proteins (9). The cornified-envelope proteins are crosslinked by several transglutaminases (TGs) to reinforce the mechanical resistance of the protein net and covalently attached to lipids, which maintain the water permeability barrier function of the skin (15). This lipid matrix is mainly composed of ceramides (CERs), free fatty acids and cholesterol (20). CERs, the dominant lipids in the SC, can be generated through *de novo* pathway, sphingomyelin hydrolysis pathway and catabolic pathway. However, most of the epidermal ceramides are produced by the *de novo* pathway, whereas ceramides used as signaling molecules are synthesized by the sphingomyelin and catabolic pathways (21).

CERs are primarily synthesized in the endoplasmic reticulum of the *stratum spinosum* and then converted to glucosylceramides (glucosyl-CER) by glucosylceramide synthase and sphingomyelins (SM) by sphingomyelin synthase, after leaving the cell via Golgi body. The glucosyl-CER and SM are stored in lamellar granules (otherwise known as lamellar bodies, membrane-coating granules or Odland bodies) and secreted later into the interface of the SG and SC. Once outside the cell, CERs are generated via hydrolysis by beta (β)-glucocerebrosidase and sphingomyelinase (21,22).

The SC is often modelled as a "brick and mortar" type of structure, where corneocytes are the bricks, and the intercellular lamellar lipid membrane is the mortar (8).

Keratinocytes are also responsible for restoring the epidermis after injury, through a process known epithelialization where cytokines and growth factors such as interleukins (IL-1, IL-6, IL-8), transforming growth factors (TGF-α, TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumor necrosis growth factor (TNF-α), interferons (IFN-α, IFN-β), and granulocyte-macrophage-colony-stimulating factor (GM-CSF or G-CSF) display a vital role (12).

Other cell types found in the epidermis include melanocytes, Langerhans cells, and Merkel cells. Melanocytes are melanin-producing neural crest-derived cells (melanoblasts) which undergo a process of migration, proliferation, differentiation, and maturation (6). The glycosylation of tyrosinase is a determinant step in melanin production (23). Melanocytes are found at the basal layer at which melanosomes are responsible for the synthesis, storage, and transport of melanin to adjacent keratinocytes in order to form perinuclear melanin caps. Cutaneous pigmentation depends on the intensity of melanogenesis and melanin distribution in keratinocytes (24). In addition to pigmentation, another essential role of melanin is to protect from harmful ultraviolet rays preventing UV-induced nuclear DNA damage (25). Langerhans cells are antigen-presenting immune cells mostly present in the *stratum spinosum*. Although, not only they can be found in all layers of the epidermis (except SC) but also in the papillary dermis (5). Merkel cells are responsible for the sensory function providing sensitivity to temperature, touch, pressure and pain (5).

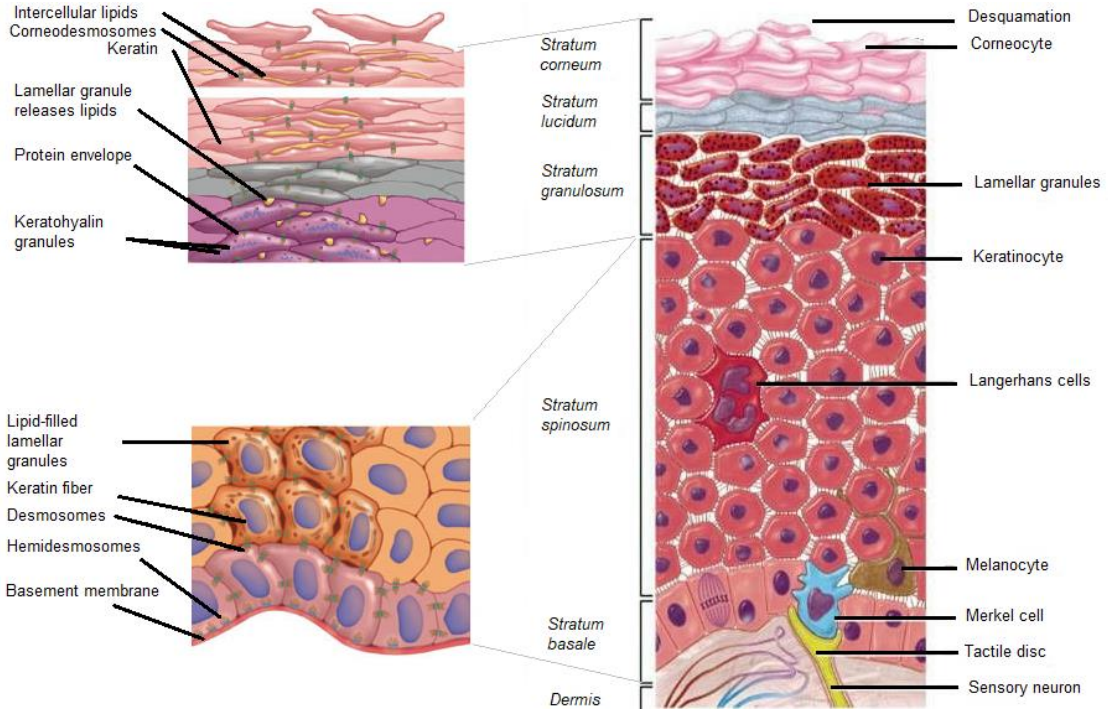


Figure 1: General structure of the epidermis. Figure was adapted from pictures extracted from http://theextremeanatomy.blogspot.com/ assessed on October 2019, and https://www.pinterest.pt/pin/539024649131831974/ assessed on October 2019.

1.2 Dermis

The dermis is divided into two distinct regions: papillary dermis, the uppermost layer of dermis located just below the dermal-epidermal junction, and the lower layer, reticular dermis.

Fibroblasts (the primary cell type), macrophages, and mast cells are included in the cell components of the dermis. Aside from these cells, the dermis is also composed of connective tissue proteins such as collagen (which provides tensile strength and mechanical resistance) and elastin (which provides elasticity) synthesize by fibroblasts. Fibroblasts are also responsible for releasing FGF-2, TGF-β, PDGF, insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) that play essential roles in wound healing, as it enables wound closure and granulation tissue formation (26).

Dermal fibers and cells are set in a matrix of macromolecules, known as ground substance, composed of glycosaminoglycans, glycoproteins and proteoglycans. Dermis anchors epidermal appendages like hair follicles, sebaceous and sweat glands (6). Besides, blood vessels present in the dermis are responsible for delivering nutrients and circulatory support, since the dermis does not have its blood supply (27). Detection of various stimuli

is performed by specialized nerve endings, such as Meissner corpuscles found in dermal papillae and *Vater-Pacini* corpuscles located at the dermal-hypodermal junction (6).

1.3 Hypodermis

Below the dermis lies a layer of adipocytes, held together by fibrous tissue, that helps insulate the body, providing a protective padding and acting as a reserve energy supply (6).

2. Changes in the structure and function of the skin

The majority of skin changes are associated with intrinsic aging processes, resulting in multiple clinical manifestations and increasing vulnerability to extrinsic factors (5,27). The oxidative stress caused by free radicals from reactive oxygen species (ROS) is strongly related to both intrinsic and extrinsic aging processes. ROS may cause damage to critical cellular components like membranes, enzymes and DNA, resulting in reduced function, abnormal transmembrane signaling, and reduced transport efficiency (1,27). Incomplete repair of this damage over time results in unusual structure stability and physiological function (27). The evolution of the structural and functional damage leads to changes of specific skin parameters that include biochemical parameters and structural parameters, including thickness, collagen content and turnover, and cell size; as well as functional parameters, such as elasticity, torsion extensibility, neuroperception, transepidermal water loss (TEWL), and proliferation rate (Table 1) (1).

Table 1: Examples of functional parameters of skin in maintaining proper epidermal barrier function (28,29).

Functional parameters	
Elasticity	Ability of the skin to come back to its normal position after being stretched**.**
Torsion extensibility	Elastic component of the skin under torsional conditions.
Neuroperception	Touch and tactile perception mediated by sensory neurons.
Transepidermal water loss (TEWL)	Amount of water that passively evaporates through skin to the external environment due to water vapor pressure gradient on both sides of the skin barrier.
Cell proliferation	Defined as the cellular growth rate or as the quantified value for the daughter cell population.

As the skin ages, decreased SC hydration is observed as a result of the reduction of the natural moisturizing factor and the lipid lamellae. Corneocyte surface area enlarges and

epidermal turnover rate generally slows. The epidermis decreases in thickness and becomes more fragile (Figure 2). Therefore, the dermal-epidermal junction shows a flattened appearance increasing the vulnerability to injury (30). The number of keratinocytes decreases as their shape changes and they become shorter and fatter (1). The senescent keratinocytes become resistant to apoptosis and may survive long enough to accumulate DNA and protein damage (27).

Blood supply to the skin is reduced as the blood vessels become more fragile, leading to compromised thermoregulation. Collagen fibers that provide structural support stiffen due to a decrease in fibroblasts and their collagen synthesis. The amount of glycosaminoglycans secreted by fibroblasts, especially HA and dermatan sulfate, also declines (27,30). Besides being involved in skin repair by promoting the proliferation and migration of keratinocytes in the re-epithelialization process and providing the framework for blood vessel formation, HA is also crucial in skin moisture due to its unique capacity in retaining water (31,32). Elastic fibers thicken with an associated higher degree of calcification. The decline of Pacinian and Meissner's corpuscles, as well as Merkel cells, increases the risk of inadvertent damage (30,33). The parallel Langerhan's cells drop out leads to impairment of cutaneous immunity (30). Reduced activity of sebaceous and sweat glands leads to thinning and drier skin and increases the vulnerability to infection (5). Blotchiness and uneven pigmentation are noticed as a result of localized overproduction of melanin follow by fewer enzymatically active melanocyte (1). The thermoregulatory function is compromised since the overall volume of subcutaneous fat typically diminishes with age, so the skin has less isolation and padding (1).

In conclusion, skin maintenance can function as primary therapy for wound prevention and starts with understanding skin anatomy, physiology, and function and identification of factors that adversely affect the wound healing process (27).

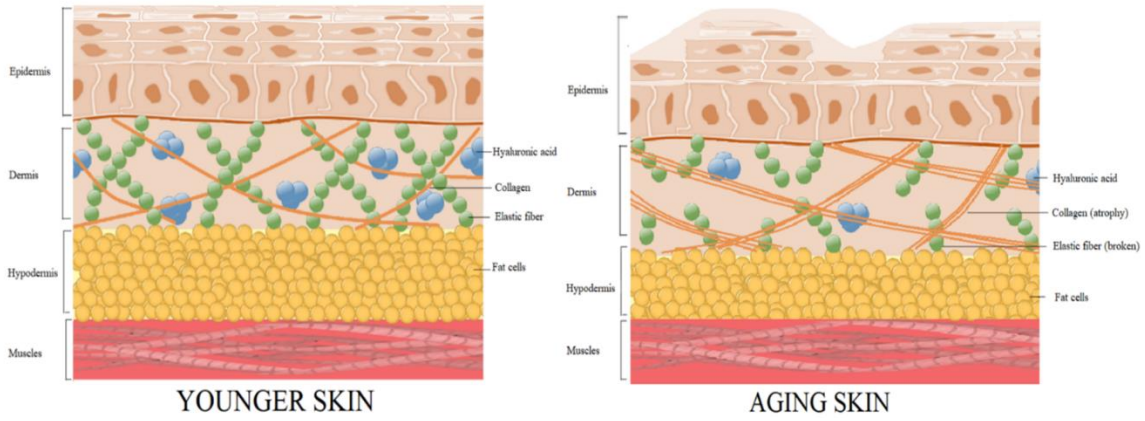


Figure 2: Effects of aging on skin structure.

3. Stages of wound healing

When the skin is damaged, a regulated sequence of biochemical events is set into motion to repair the injury. The process of skin repair is divided into four sequential phases: (1) hemostasis, (2) inflammatory, (3) proliferative (including re-epithelialization, granulation tissue formation, and neovascularization), and (4) remodeling (34).

3.1 Hemostasis

In damaged skin, the exposure type I collagen and leakage of blood constituents activates the clotting cascade and homeostasis (34,35). In response to the damage of microvessels, local vasoconstriction occurs near the injury point under the effects of mediators (such as prostaglandins, and thromboxane), reducing blood loss (36).

Activated platelets subsequently start to aggregate to the damaged surfaces - platelet adhesion- in order to form a clot. Platelet adhesion is mediated by von Willebrand Factor, previously formed and stored in circulating platelets (37). Adhered platelets undergo degranulation by proteins such as thrombin, releasing many soluble mediators from dense granules, known as α granules. Additional platelets, stimulated by ADP (adenosine diphosphate) released by activated platelets, arrive and begin to stick to each other – platelets aggregation (38). Epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), PDGF, TGF–α,β, FGF-2 and IGF-1 are secreted by aggregated platelets from α granules, which activate and attract other response cells such as neutrophils, monocytes/macrophages, smooth muscle cells, fibroblasts and endothelial cells (26,34). Platelet degranulation also leads to the release of serotonin, histamine, glycoproteins (e.g.,

fibrinogen, fibronectin, thrombospondin and vitronectin) and several inflammatory mediators, such as IL-1α, IL-1β, IL-6, IL-8 and TNF-α (34,39,40). Besides being responsible for initiating the coagulation cascade and releasing growth factors, the platelet degranulation is also responsible for the activation of the complement cascade. Fibrinogen is converted to fibrin, which along with platelets, plasma fibronectin, vitronectin and thrombospondin consolidates the primary platelet clot, forming a stable hemostatic plug within minutes (3,12,37).

After hemostasis is achieved, prostaglandins and leukotrienes are released by local endothelial cells (12). Therefore, increased blood flow and altered vascular permeability along with numerous vasoactive mediators and chemotactic factors mediated by the activated coagulation cascade, complement pathways, and parenchymal cells continue to increase the migration of inflammatory cells to the wound (34). This process is briefly described in Figure 3.

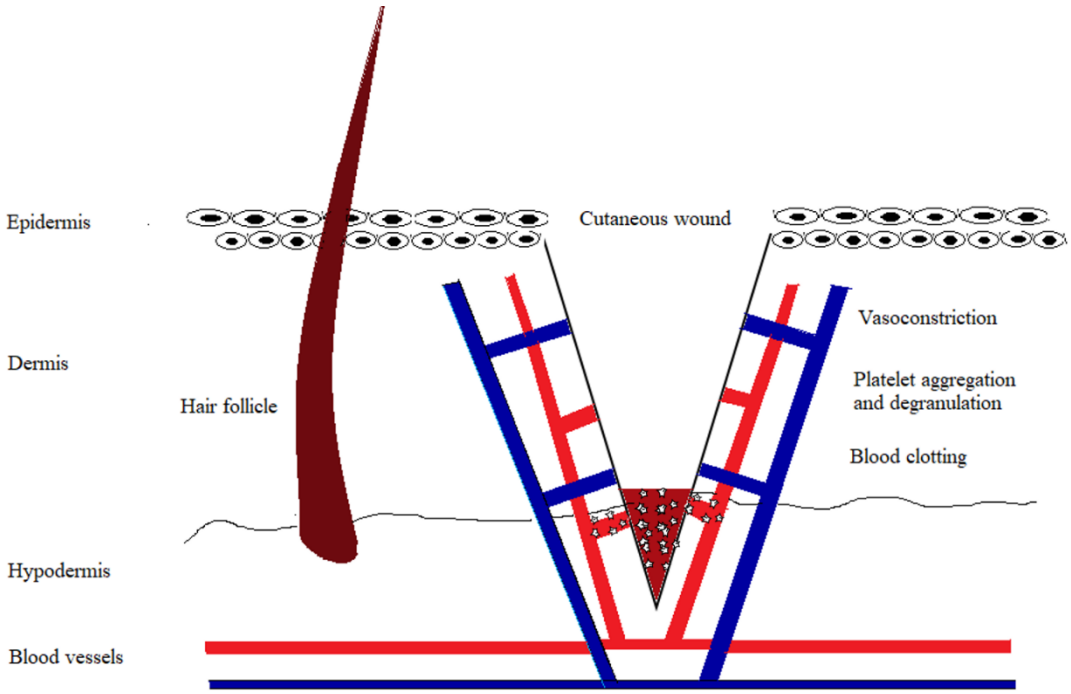


Figure 3: Hemostasis phase of wound healing.

3.2 Inflammatory phase

The second phase of wound healing starts within 24 hours when neutrophils arrive at the wound site to scavenge injured cells, damaged matrix components, pathogens and bacteria as seen in Figure 4 (12). Neutrophils can upregulate gene expression of chemokines such as TNF-α, IL-1β, IL-6, IL-8, chemokine (C-X-C motif) ligand 2 (CXCL2), and monocyte chemoattractant protein-1 (MCP-1/CCL2) that act as

chemoattractants for macrophages, T cells, and additional neutrophils (39). Neutrophils produce a wide variety of proteases (elastase, cathepsin G, proteinase 3 and a urokinase-type plasminogen activator) and antimicrobial substances (cationic peptides and eicosanoids) and can phagocytize bacteria by forming neutrophil extracellular traps (NETs) (41–43).

Monocytes, attracted by chemotactic agents including TGF-β, PDGF, MCP-1, fibronectin and other extracellular matrix (ECM) protein fragments, migrate into the wound and differentiate into mature macrophages (12,34). Macrophages continue clearing debris through phagocytosis of apoptotic cells, bacteria and foreign materials and through the release of the plasminogen activator, they cause the dissolution of the fibrin clot (26). In addition, macrophages secrete pro-inflammatory cytokines: IL-1, IL-6 and TNF-α, and a variety of growth factors such as bFGF, TGF-α, TGF-β, PDGF, VEGF, EGF and heparin-binding EGF-like growth factor (HB-EGF) (26,34,44). Both neutrophils and macrophages release high levels of ROS (45).

The growth factors and cytokines released during this phase, as also interleukins and interferons, transcriptionally activate a group of calcium-dependent zinc-containing enzymes called matrix metalloproteinases (MMPs) (46). MMPs, which are expressed by inflammatory cells, fibroblasts, endothelial cells, and keratinocytes have an essential role in leukocytes migration, angiogenesis and re-epithelialization (47). Tissue inhibitors of metalloproteinases (TIMPs) are the principal endogenous inhibitors of MMPs, and an imbalance between MMPs and TIMPs ratio can result in impaired wound healing (44).

The phagocytosis of neutrophils has been described to influence the transition from pro-inflammatory macrophages, traditionally referred to as "M1" macrophages to M2 anti-inflammatory macrophages, changing their phenotype and cytokine profile expression (from an inflammatory profile to a resolutive one) (45). These findings suggest that the switch from M1 to M2 phenotype stimulates keratinocytes, fibroblasts, and angiogenesis to promote tissue repair, facilitating the transition to the proliferative phase (48).

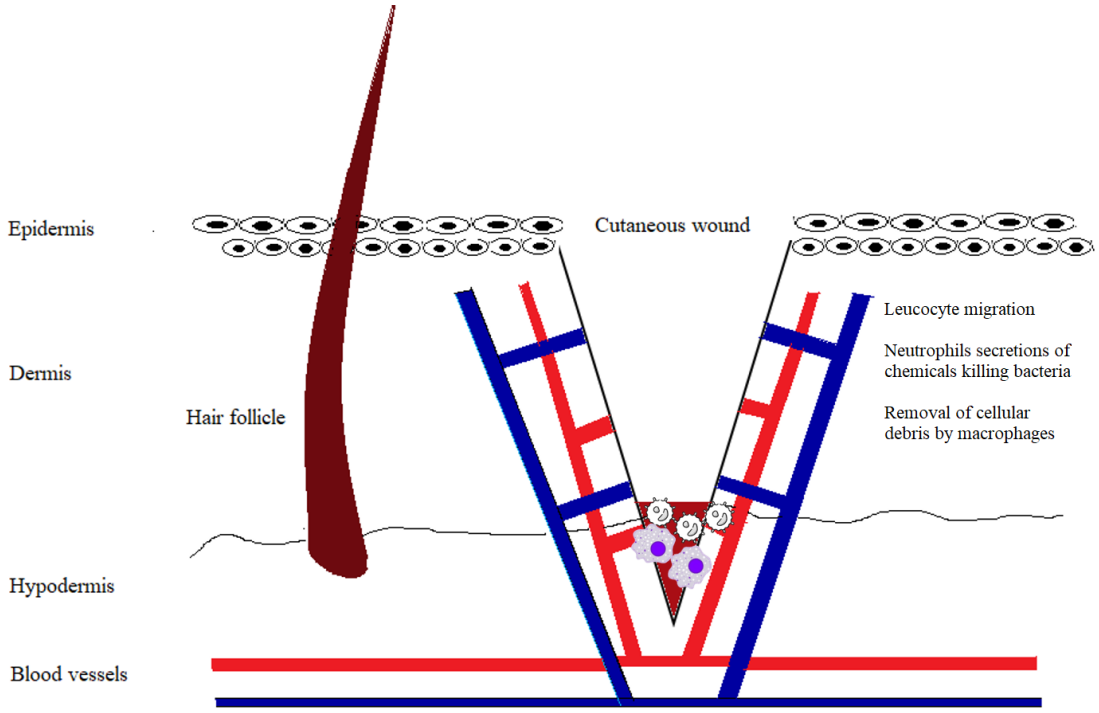


Figure 4: Inflammatory phase of wound healing.

3.3 Proliferative phase

During the proliferative phase (occurring from day 4 to day 21), the temporary fibrin matrix is rebuilt with granulation tissue mainly made up of type-III collagen, elastin, proteoglycans, glycosaminoglycans (e.g., hyaluronic acid), and non-collagenous glycoproteins, as the fibroblasts migrate and proliferate to the wound area (26,49). This phase is represented in Figure 5. In the early stage, the major components of the granulation tissue matrix are hyaluronic acid (HA) and fibronectin. Over time, the amount of HA decreases while is observed an increased synthesis of collagen accompanied by gradual depletion of fibroblasts through phagocytosis (42).

The provisional matrix provides a scaffold for production of new blood vessels (through a process referred as angiogenesis or neovascularization), necessary to provide nutrition and oxygen to help maintain the granulation tissue bed tissue and allowing efficient drainage of metabolites. Pro-angiogenic factors stored in platelets and inflammatory cells are responsible for endothelial cell activation (50). FGF, released by damaged endothelial cells and macrophages, and VEGF, which is released by keratinocytes and macrophages are examples of proangiogenic factors (Table 2) (51). The activated endothelial cells secrete MMPs which are responsible for the degradation of ECM components and, subsequently, contribute to angiogenesis.

Table 2: Examples of angiogenic stimulators and inhibitors.

Stimulators	Inhibitors
aFGF (FGF-1)	Thrombospondin-1
bFGF (FGF-2)	TIMPs
TGF-α	IFN-α/β/γ
TGF-β	Angiostatin
PGE2	Endostatin
TNF-α	
VEGF	
EGF	

Multiple mediators such as growth factors (EGF, KGF, and TGF-α), cytokines, integrins, keratins, MMPs, chemokines, and extracellular macromolecules stimulate the keratinocytes to proliferate and migrate into the wound, initiating the process of re-epithelialization (9,52).

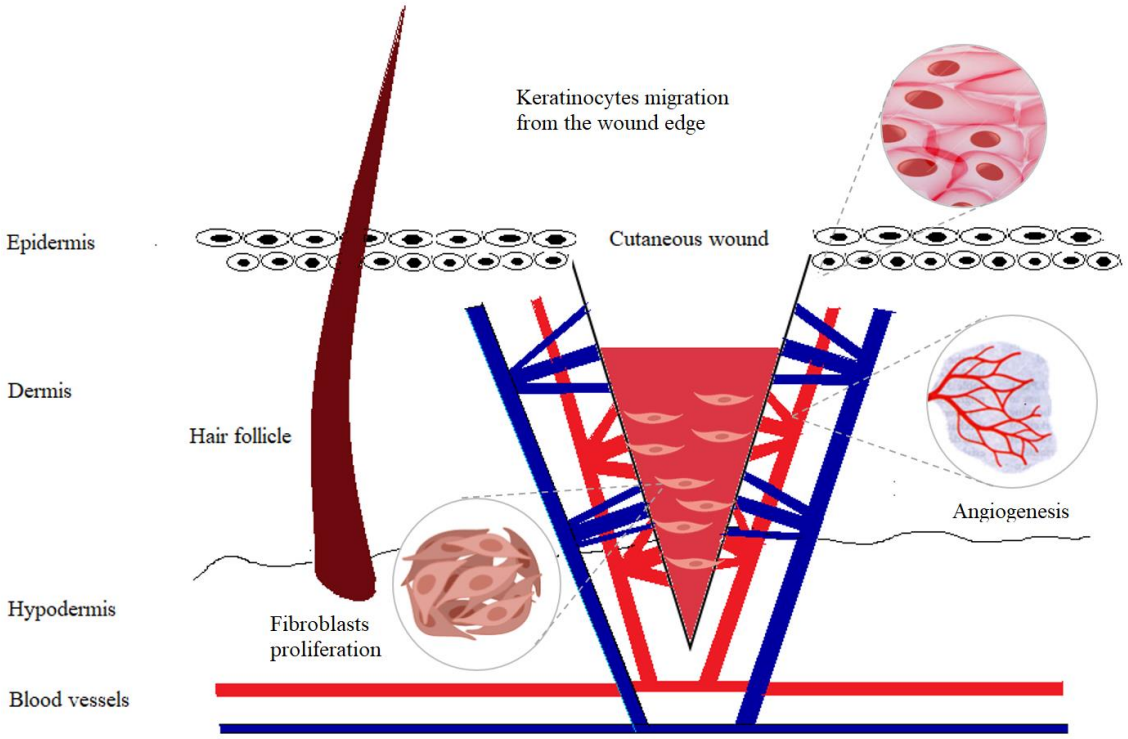


Figure 5: Proliferative phase of wound healing.

3.4 Remodeling phase

Remodeling phase is characterized by the transition from granulation tissue to scar formation, specifically the replacement of type III collagen, which is prevalent during

proliferation, to type I collagen (3). Therefore, originally disorganized collagen fibers are rearranged, cross-linked and aligned through the continued synthesis and catabolism of collagen by MMPs, reducing scar thickness and strengthening the matrix (12).

As seen in Figure 6, during this phase (which begins after day 21, and lasts between weeks-months-years depending on patient and wound-related complicating factors such as patient comorbidities and wound infection status), excess matrix materials that had been used to repair the wound (including blood vessels formed in the granulation tissue) but which are no longer needed are removed by apoptosis or programmed cell death (3,42,53). After exposure to TGF-β and mechanical loading signals, fibroblasts are activated and differentiate into myofibroblasts, which contract by using smooth muscle type actin-myosin complex, called alpha-smooth muscle actin (α-SMA), causing wound contraction and therefore facilitating wound closure (54).

Table 16 (in annex) summarizes the growth factors involved in wound healing process.

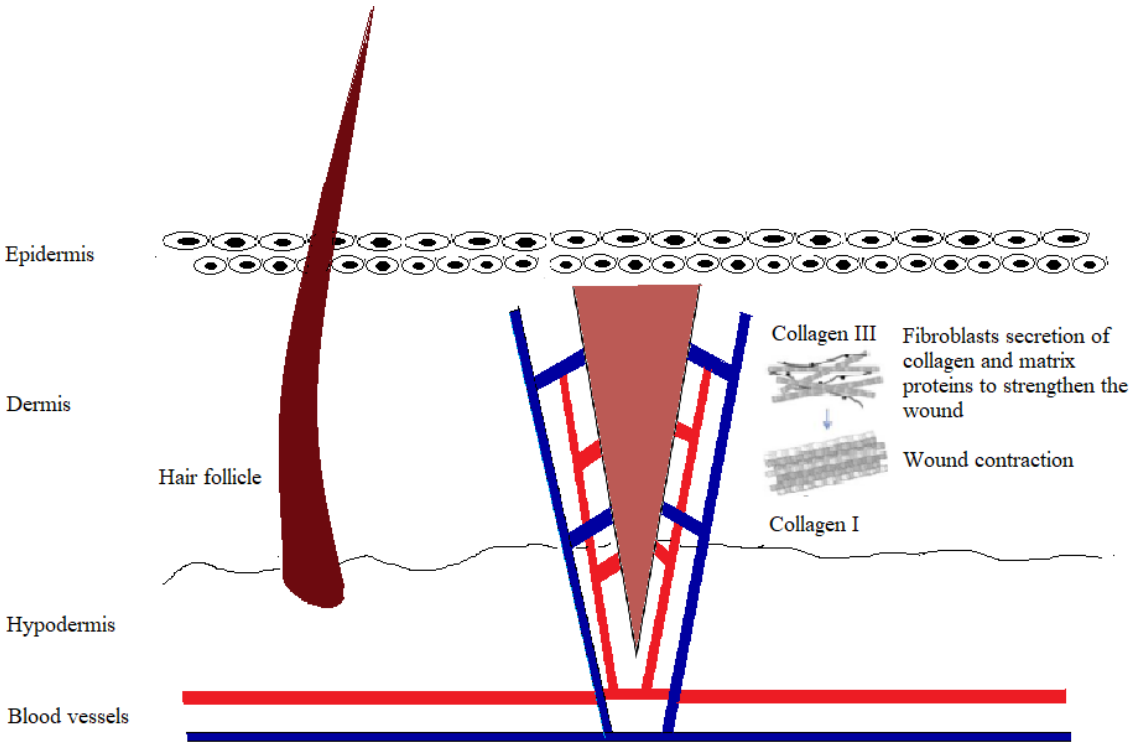


Figure 6: Remodeling phase of wound healing.

4. Impaired wound healing

Skin repair is the result of dynamic and interactive processes that require the coordination of many mediators such as inflammatory cells, soluble mediators, many

resident cells and ECM molecules, such as fibronectin, glycosaminoglycans, proteoglycans, thrombospondins, tenascin, vitronectin, and collagens (26). Thus, impaired healing is observed when wounds fail to progress through typical, orderly and timely phases of wound healing, being abnormal scarring (hypertrophic and keloid scars), and non-healing wounds (ulcers or chronic wounds) the major outcomes (3, 26). The first situation is caused by an overproduction of immature collagen, in contrast to the other case, which is characterized by prolonged inflammation. In this thesis, we focus specifically on non-healing wounds.

In healthy individuals, epidermal barrier repair is highly efficient (Figure 7). However, the prevalence of delayed wound healing increases as certain factors are present, as previously mentioned (55).

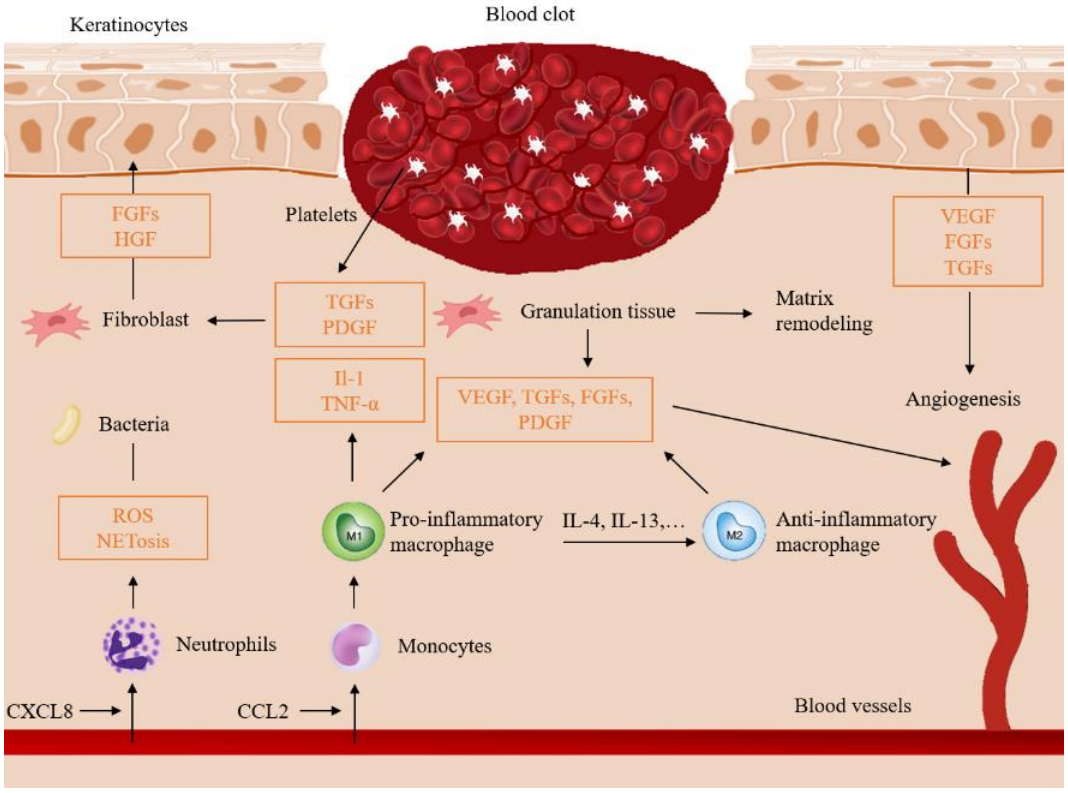


Figure 7: The acute healing process.

In fact, wounds have an immense financial burden on health-care systems worldwide. Globally, the annual cost for wound care was an average of $2.8 billion in 2014 and is expected to rise to $3.5 billion in 2021. As a result, there is a significant demand for wound care products and the global market is estimated to exceed $15 billion by 2022 according to the 2018 market research report (56). With approximately 300 million chronic and 100

million traumatic wound patients worldwide, the impact of chronic wounds is a universal aspect of medical care (57). Chronic wounds not only contribute major costs to health-care systems but also have devastating consequences for patients.

Chronic wounds are often characterized by low levels of mitotic activity, elevated inflammatory cytokines and proteases (including elastase), small growth factors activity and nearly senescent fibroblasts, as seen in Figure 8 (47,58).

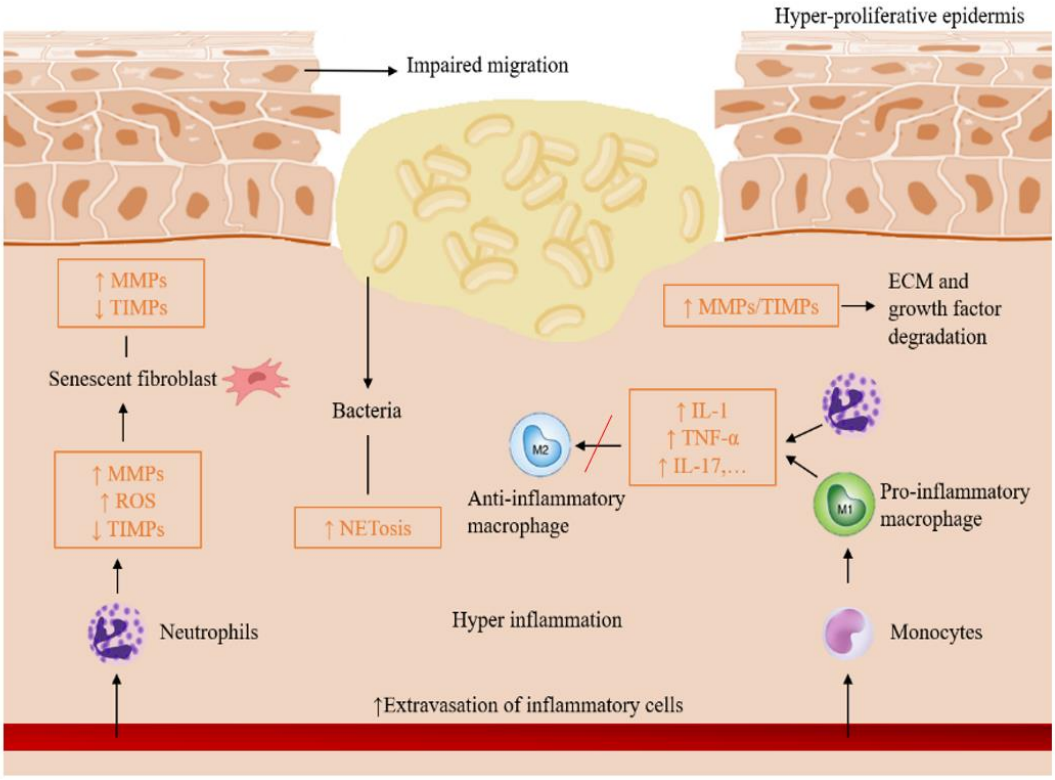


Figure 8: The non-healing wound process.

It has been showed that the amount of inflammation determines wound fibrosis. Thus, the lack of intrauterine inflammation is mentioned as evidence for the remarkable ability of scarless wound healing in the fetus (42). Persistent inflammation, such as that seen in chronic wounds, is characterized by abundant neutrophil infiltration, with an associated increase of the ROS levels due to a reduced phagocytic capacity of macrophages (48,59). ROS have a vital role in the defense against pathogenic microorganisms; however, high levels can cause tissue damage by adding more oxidative stress to the wound and, subsequently increasing the MMPs levels (44). It is not entirely clear if whether the prolonged open wound with a predisposition to infection is causal of the chronic inflammation, or vice versa, or both (60). High levels MMPs lead to excessive degradation of ECM constituents and disruption of cell migration, as evidenced for chronic wounds

(44). The ECM deterioration associated with the poorly respond of fibroblasts to growth factors, contribute to faulty granulation tissue (52).

Although the influx of innate immune cells into chronic wounds may have a negative impact on the repair process resolution, some immune cell lineages like Langerhans cells have been associated with better healing outcomes (60).

Wound healing is further complicated by the presence of biofilms (mono- or polymicrobial) responsible for the production of their own proteases, which can inhibit keratinocyte migration and re-epithelialization (9). Initially, the microorganisms tend to attach to the surface. However, their initial attachment is reversible, and they are still susceptible to antibiotics. Further on, they become more firmly attached and begin to secrete a mucopolysaccharide matrix composed of microbial and host molecules, which will lead to a mature biofilm characterized by tolerance to antibiotics and immune control (61). Despite their hyperproliferative state at the chronic wound edges, keratinocytes are unable to migrate due to their incomplete activation and differentiation (62). High levels of proinflammatory cytokines (IL-1 and TNF-α) are expected as an attempt to cause a fibroblast response, leading to increased secretion of MMPs/reduced secretion of TIMPs. Microvessels are surrounded by fibrin cuffs composed of fibrin and plasma proteins, resulting in chronic hypoxia and impaired micronutrient delivery and further tissue damage (50,60).

5. Skin injuries

The skin can be damaged by abrasion, avulsion, laceration, puncture, burn, friction, constant moisture, unrelieved pressure, and shearing forces, as seen in Figure 9.

An incision wound refers to a clean cut in the skin caused by a sharp object. Abrasions, commonly known as scrapes, occur when the skin is rubbed against a hard or rough surface and can range from mild to severe. The most severe abrasion it is also known as an avulsion wound where there is a partial or complete tearing away of skin and the tissue beneath. A laceration refers to a deep cut or tearing of skin caused by knives and other sharp tools. A pointed and sharp object can also cause puncture wounds, and the result is often a small hole. However, it can be deep enough to involve nerves, tendons, and organs. Burns, heat, and cuts are common causes of wounds (63).

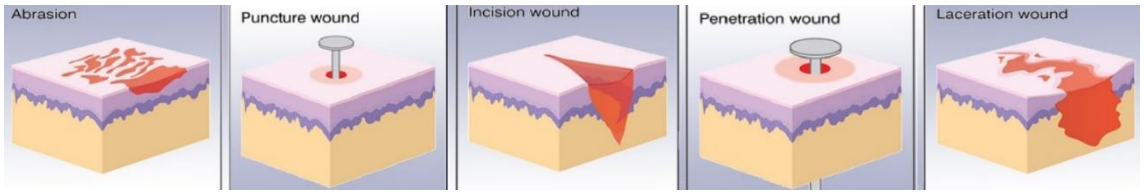


Figure 9: Different types of wounds extracted from name et al. (64).

There are different types of burns, such as thermal burns caused by fire, steam, hot objects or hot liquids; electrical burns caused by contact with lightning or electrical energy sources; chemical burns that can be caused by strongly acid or alkali substances, detergents or solvents; radiation burns due to prolonged exposure to ultraviolet rays of the sun, X-rays or during radiotherapy; and friction burns that occur when skin repeatedly rubs against another surface or is scraped against a hard surface. Burns are categorized into degrees, first, second, and third. First-degree burns damage the outermost layer of the skin, the epidermis. Second-degree burns damage both the epidermis and the dermis. Third-degree burns are more severe, as they destroy both layers of the skin. Hair follicles, sweat glands, and other tissues can be injured as well. Technically, there is also a fourth degree of burn, which involves the tendons, muscles, and bones (65).

Skin ulcerations can have several causes and thus different treatments. Friction, shear, moisture and immobility among others are identified as contributing factors to pressure ulcers. The compressed blood supply and the obstruction of lymphatic flow lead to ischemic tissues and accumulation of metabolic products, proteins, and enzymes. Other etiologies for skin ulcers include the presence of venous disease/insufficiency, arterial disease or mixed (venous and arterial diseases) and diabetic neuropathy. Venous ulcers represent the most frequent type of ulcers (40,66). Examples of these ulcers are shown in Figure 10.

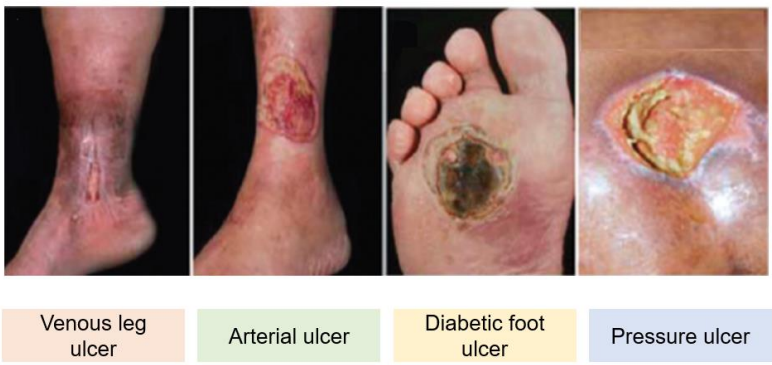


Figure 10: Different types of ulcers. Images extracted from publish data (63).

6. Approaches to cutaneous wound healing: present and future

Nowadays, several approaches are sought in order to improve cutaneous wound healing, which include both conventional and advanced technologies. In addition to conventional technologies, such as skin grafts and wound dressings, newer approaches have been developed, including gene targeting, stem cell treatment, soluble molecules, tissue engineering, etc. These procedures are now described in the following sections.

6.1 Wound Dressings

Dressings are designed to be breathable, allowing gas exchange between wounded tissue and environment and, consequently, protecting the periwound skin from maceration and promoting autolytic debridement. A dressing acts as a barrier, reducing the risk of infections and providing thermal insulation and, thus, prevents further complications. It must also be capable of maintaining the stable moist wound environment. Treatment of chronic wounds includes different types of wound dressing with a variety of aims including maintenance of a moist environment (films, foam, hydrogel and hydrocolloid as described in Table 3), reduction in bacterial load and infection (containing antibiotics, antiseptics, and natural antimicrobials) or dressings aiming to support healing that contain collagen, cellulose and others (67).

Table 3: Types of wound dressings (57,67–69).

Type	Description	Advantages	Disadvantages	Indications
Films	Usually composed of a thin sheet polyurethane coated with an adhesive	Good gases-permeability, impermeable to bacteria and fluids, easy wound monitoring through film transparency, and less maceration and painless.	Difficult handled, adherence to wound bed, non-absorbent allowing wound exudates accumulation, easy bacterial invasion and infection, and impermeable for proteins and drugs.	Recommended for re-epithelializing wound, superficial wound and shallow wound with low exudate.
Foams	Made of microporous upper layer and sponge-like porous sublayer.	High absorbent without compromising the moist environment, huge porosity, provide thermal insulation, non-leakage against bacterial invasion, very easy used, and costless.	Very adherent, forming opaque layer which complicates wound monitoring, semi-permeable for gases, poor stability, non-applicable for low exudating wounds, dry wound, and dry scars.	Suitable for lower leg ulcers and moderate to highly exudating wounds, and granulating wounds.

Hydrogels	Insoluble hydrophilic polymer-based with high water content (70-90%).	Absorb some exudate, non-adherent, easily removed from wound, accelerate the healing, pain and inflammatory reduction and costless.	Limited capacity to absorb exudate, which could lead to exudate accumulation causing wound maceration, poor bacterial barrier, low mechanical strength, demanding a secondary dressing.	Used for dry chronic wounds, necrotic wounds, pressure ulcers and burn wounds.
Hydrocolloids	Contain gel-forming agents (carboxymethyl cellulose, gelatin and pectin) combined with other materials like elastomers and adhesives.	Absorption of some exudate, easily removed by saline or sterilized water, non-adherent, painless, high density, waterproof, permeable to water vapor and impermeable to bacteria, they create an acid pH environment at the wound site.	High leakage exudates, not indicated for neuropathic ulcers or highly exudating wounds. They can be cytotoxic and display an unpleasant odor. They can have low mechanically stability.	Used on light to moderately exudating wounds such as pressure sores, minor burn wounds and traumatic wounds.
Alginates	Primarily made of seaweed derivatives, such as calcium or sodium alginate.	High absorbent, non-adherent, high mechanical stability, easily removed by saline solution, good bacterial barrier, hemostatic property.	Very cost, difficult handled and require secondary dressings because it could dehydrate the wound which delay healing.	Suitable for moderate to heavy drainage wounds.

6.1.1 Stem cells therapy

Stem cells therapy is another promising approach that has several advantages as described in annex (Table 17). However, stem cells have only limited viability, which means that it is not guaranteed the engraftment of transplanted cells in a direct injection.

In a recent study, a combination of HA and adipose-derived stem cells (ASCs) was applied and the injured area was then covered by an acellular dermal matrix dressing, revealing to be able to stimulate wound healing by reducing inflammatory response, promoting proliferation and differentiation of fibroblasts, and enhancing angiogenesis and re-epithelialization in burn wound model in rat (70).

In a murine model, a heparinized HA hydrogel was used for delivery of amniotic fluid-derived mesenchymal stem cells (AF-MSCs) via bioprinting. This study revealed an improved wound closure with enhanced re-epithelialization and vascularization (microvessel density significantly greater with $p < 0.01$) and increased production of ECM (71).

A hydrogel loaded with bone-marrow derived mesenchymal stem cells (BM-MSCs) confirmed to enhance fibroblast and keratinocytes proliferation. The BMSCs group also exhibited higher TGF-β1 and bFGF secretion and reduction of inflammatory responses at wound sites (72).

Some researchers investigated the effectivity of a spinner flask suspension culture system for the maintenance of umbilical cord tissue-derived MSCs (UC-MSCs), UCX® spheroids which successfully mimicked the native cell microenvironment, enhanced keratinocyte/fibroblast migration and proliferation showing complete re-epithelialization, higher vascularization levels and elastin production when compared to the controls (73).

The first Portuguese patent with stem cells refers to a 3D fibrin gel with umbilical cord blood-derived hematopoietic stem cells ($CD34^+$ cells) co-cultured with $CD34^+$-derived endothelial cells for the treatment of chronic wounds (74).

6.1.2 Growth factor therapy

As mentioned before, there are several growth factors, which are known to be involved in the wound healing process. Growth factors have been tested as topical treatments to enhance wound healing. However, the exogenous application of GFs still has limited success in wound care due to challenging features like described in Table 17. Only a recombinant human PDGF (rhPDGF) topical hydrogel has been approved in EU for clinical application in DFUs, commercially available as Regranex® Gel, an aqueous-based sodium carboxymethylcellulose gel containing 0.01% becaplermin (75). In the meantime, the Regranex® authorization has been withdrawn at the request of the marketing-authorization holder (76).

6.1.3 Nucleic acid delivering

Gene-mediated therapeutic delivery involves the localized transfection of nucleic acids into cells with the intention of altering gene expression. Due to several advantages, gene therapy is a new and emerging technology for wound repair (Table 17).

Porous HA-MMP hydrogels with encapsulated VEGF plasmid DNA were used to investigate scaffold-mediated gene delivery for local gene therapy in diabetic wound healing in mice. The porous hydrogels revealed an indistinguishable better wound closure by day 14 compared in n-pore hydrogels; however, the degree of gel degradation and DNA release did not seem to be faster enough to enhance angiogenesis (77).

6.1.4 Cell encapsulating

Hydrogels have gained considerable attention as suitable materials for cell encapsulation because of inherent properties related to their biocompatibility and crosslinked nature which can modulate the loading and release of the encapsulated cells (78).

Constant insulin-secreting cells were encapsulated in poly(ethylene glycol) diacrylate hydrogel microspheres demonstrating significant acceleration in wound closure by postoperative day 7 (5.07% – 19.5%), increased keratinocyte migration *in vitro* and revealing an epidermis significantly thicker in treated diabetic mouse model (42.7 – 10.37 mm) compared with control (29.98 – 7.96 mm) (79). Some studies have shown the potential effects of insulin in growth, proliferation, secretion, and migration of keratinocytes, endothelial cells, and fibroblasts, thus, improving re-epithelialization and angiogenesis (79,80).

Another example is related to an *in vivo* study on the incisions of rat skin reported that the use of a fibroblast-encapsulated PEG-b-poly(L-alanine) hydrogel which significantly accelerated wound healing by inducing cell proliferation, collagen production and re-epithelialization compared to the controls treated groups (81).

6.1.5 Drug or antibiotic-loaded dressings

With the growing problem of antibiotic resistance, new antimicrobials isolated from natural products, modified antibiotics, or new dosage forms like nanoparticles (NPs) can be used as alternatives to conventional antimicrobials. Between the two possible forms of administration, the advantages of topical over systemic are higher availability at the site of infection, fewer systemic adverse effects, and a lower incidence of microbial drug resistance (82). S. *aureus*, P. *aeruginosa* and E. *coli* are common causes of delayed healing and infection in both acute and chronic wounds (83,84). Additionally, C. *albicans* is the prevailing cause of burns infections (85).

Electrospun fibers based on thermoresponsive polymer poly(N-isopropylacrylamide), poly(L-lactic acid-co-ε-caprolactone) (PLCL) and ciprofloxacin exhibited good antibacterial activity with inhibition zones of 5.19 – 5.24 cm for E. *coli* and 5.13 – 5.17cm for S. *aureus*, performing better wound closure (showing wound areas of 6.50 ± 3.14 % and 7.71 ± 3.32% for the ciprofloxacin-loaded fibers) than those containing only PLCL and ciprofloxacin (14.36 ± 2.83 %) (86).

Sponges formed by grafting amoxicillin onto regenerated bacterial cellulose (BC) expressed highly effective antimicrobial activities against E. *coli*, C. *albicans* and S. *aureus,* exhibiting strong impact in bacterial cell membrane/wall integrity resulting in cytoplasm leakage. The functionalized sponges showed good wound healing ability, displaying a wound closure rate of 80% on day 12. The control group displayed only 65% (87).

The antimicrobial activity of composite films and wafers comprising polyox/carrageenan (POL-CAR) and polyox/sodium alginate (POL-SA) loaded with diclofenac (DLF) and streptomycin (STP) was tested against pathogenic bacterial species and compared with marketed silver dressings. For S. *aureus* and E. *coli*, the authors determined minimum inhibitory concentration values (MIC, defined as the lowest concentration to inhibit visible growth) of STP between 4 to 8 mg/L, whereas values ranged from 8 to 16mg/L for P. *aeruginosa*. For DLF, the MICs against P. *aeruginosa* was >512 mg/L and 256–512mg/L for E. *coli* and S. *aureus*, respectively. POL-SA dressings showed higher effectiveness against the three bacterial strains compared to POL-CAR, while the STP and DLF loaded films and wafers showed greater antibacterial activity than marketed silver-based dressings most likely due to their synergetic effect. The films also showed greater antibacterial efficacy than wafers. These researchers suggested that the higher loading capacity of the wafers was responsible for more considerable amounts of sodium sulphate which decreased the hydration capacity and, thus, affected drug diffusion (88).

6.1.6 Antiseptic dressings

Antiseptic dressings exhibit a broad antimicrobial spectrum and residual anti-infective activity on wounds; however, they often show dose-dependent cytotoxicity to the host cells including keratinocytes, fibroblasts, and leukocytes (82).

Polyhexamethylene biguanide (PHMB) has been successfully incorporated into a range of wound products, such as solutions and gels (e.g. Prontosan®, which contains 0.1% solution of PHMB) and wound dressings (e.g. Kendall™ AMD Antimicrobial Foam, which are impregnated with 0.5% PHMB). Specifically, PHMB binds to the plasma membrane resulting in leakage of potassium ions and cytosolic components and eventually cell death by necrosis. It is antiseptically effective against E. *coli*, S. *aureus* and S. *epidermidis* (82,89).

Chlorhexidine products have been used in wound care for many years. Bactigras® (a paraffin gauze containing 0.5% chlorhexidine acetate) and Irrisept® (a

chlorhexidine gluconate solution 0.05%) are frequently used as chlorhexidine-based preparations. One of the possible mechanisms underlying its antibacterial activity relate to the nonspecific binding of the negatively charged membrane phospholipids of bacteria to the positively charged chlorhexidine, which causes rupture of the cell wall and therefore cytoplasm leakage, triggering cell death (82).

The two most commonly used iodine compounds in modern wound dressings are povidone iodine and cadexomer iodine. Povidone iodine preparations include dressings such Inadine® and solutions such as Betadine®. The benefit of povidone iodine use is controversy due to perceived issues with toxicity and delayed healing. The efficacy of cadexomer iodine has been demonstrated. However, the mechanism of action is not totally understood. Examples include Iodosorb® (0.9% gel) and Iodoflex® (0.9% dressing) (82).

Introduced more than 20 years ago, octenidine is available as Octenilin® gel and Octenilin® solution (0.05% octenidine). Due to its low cytotoxicity and high antimicrobial efficacy, octenidine is a good candidate for preventing or treating wound infection or colonization while not affecting wound healing. To further validate this data, a randomized clinical trial aimed to evaluate the effects of octenilin wound gel on bacterial colonization and re-epithelialization using superficial skin graft donor sites. Octenidine showed no undesirable interaction with wound re-epithelialization, but still was able to be highly effective against E. *coli* or S. *aureus* (90).

6.1.7 Negative pressure dressings

Negative pressure wound therapy (NPWT), also called vacuum-assisted closure (V.A.C.), is an available tool in the management of heavily contaminated ulcers. PWT involves the application of a dressing (that facilitates pressure transmission within the wound) to which a computerized unit is attached to intermittently or continuously convey negative pressure to promote wound healing. Wound healing is thought to be assisted by provision of a moist environment, an improved exudate removal which help establish fluid balance, a potential decrease in wound bacterial load and an increase in the blood flow and tensile strength. In addition, NPWT can lower costs by reducing treatment duration and health care resource use (Table 17). The dressings used for the technique include open-cell foam dressings and gauze with 400–600 μm pores sealed with an occlusive dressing intended to contain the vacuum-generating device with a collection reservoir. The open pore structure enables uniform distribution of negative pressure at the wound site and helps exudate removal. NPWT systems such as PICO (single use) and RENASYS are available

and both CE marketed. Dressings such as V.A.C.® WhiteFoam (PVA foam), GranuFoam Silver and V.A.C.® GranuFoam™ (PU foams) are FDA-approved (91–94).

6.1.8 Oxygen delivering

Hyperbaric oxygen therapy (HBOT) has been used as an adjunct in wound healing and typically involves placing the patient in a sealed chamber where 100% oxygen is delivered at high atmospheric pressure (1.5 to 3 atmospheres absolute) for 60–120 min. HBOT increases the oxygen saturation of plasma, dramatically increasing the partial pressures of oxygen in tissues throughout the body. The improved oxygen gradient between the wound dead space and the periwound increases fibroblast proliferation and collagen deposition, improves re-epithelialization, angiogenesis, and capability of bacterial killing.

The application of HBOT is particularly advantageous in patients with diabetic foot ulcers where it is associated with greater decrease in wound size and greater rate of complete ulcer closure and could significantly reduce the risk of major amputation. The major advantages and disadvantages of this therapy are summarized in Table 17. Other possible treatments to overcome tissue hypoxia is topical oxygen treatment in form of oxygen–releasing wound dressings, which are used as a more cost-effective, portable, and rapid possibility of promoting wound healing with reduced risks of systemic oxygen toxicity. The oxygen is stored between an occlusive upper layer and a lower permeable film, which allows the dressing to supersaturate the wound fluid using microbubbles. For example, Oxyzyme and Iodozyme are commercially available hydrogels developed to support the wound healing process by releasing oxygen and impeding microbial growth through the release of iodine (91,92).

6.1.9 Biomaterials

Different biopolymers have been used for their wound healing properties and they can be divided into synthetic or natural. Naturally derived biopolymers may typically be classified into two categories: polysaccharide-based (e.g., HA, cellulose, starch, alginate, and chitin and its derivative, chitosan, among others) or protein-based (e.g., collagen, gelatin, keratin, etc.). Some of the most frequently used biomaterials in wound management, their properties and commercial examples are summarized in Table 4.

Table 4: Most frequently used natural biomaterials in wound management, their properties and commercial examples (68,89,95–100).

	Biomaterial	Properties	Examples of commercial products
Polysaccharide-based biomaterial	Starch	One of the most common and cost-effective polysaccharides. Renewable and biodegradable, poor hydrophilicity, but is very hygroscopic and binds water reversibly. Insoluble in common solvents and biosynthesis and cellular processing highly complex.	Aquaform® hydrogel
	Chitin and chitosan	Hemostatic, low immunogenicity, biocompatible, mucoadhesive properties, biodegradable, antimicrobial activity with high resistance against environment conditions.	ChitoHeal Gel
	Cellulose	Versatile biomaterial with a unique nanostructure high mechanical strength, and remarkable swelling properties. Biocompatible, biodegradable, antimicrobial, hypoallergenic and nontoxic.	Dermafill™ Cellulose Membrane Dressing
	Hyaluronic acid	Naturally occurring, non-immunogenic, anionic, non-sulfated glycosaminoglycan. Biocompatible, biodegradable. Lubricative and water absorptive. Has bacteriostatic and anti-adhesive properties.	Hyalofill®-F Biopolymeric Wound Dressing
Protein-based biomaterial	Collagen	Cost-effective, good biocompatibility, thickening and water binding capacity, controllable biodegradability, appropriate mechanical strength and flexibility. However, it has high permeability to bacteria and microorganisms.	Fibracol® Plus Collagen Wound Dressing with Alginate
	Gelatin	Gelatin lacks antigenicity in comparison to its precursor (denatured collagen) and is biocompatible, biodegradable and non-immunogenic.	Gelfoam® Absorbable Gelatin Sponge
	Keratin	Good structural integrity and solubility, biocompatibility, controllable biodegradability, bioactivity with high water's retention capacity.	KeragelT®
Natural antimicrobials	Honey	Good antimicrobial properties due to its low pH, hygroscopic nature, and peroxide-containing compounds.	MediHoney
	Curcumin	Shows antioxidant and radical scavenging properties, as well as antimicrobial and anti-inflammatory activities, however, has poor aqueous solubility, low bioavailability and rapid metabolism.	Still during development

The main qualities of natural biopolymers over synthetic biopolymers are their versatility, biocompatibility, sustainability and intrinsic structural resemblance of native tissue ECM. However, their weak mechanical strength and inconsistency in compositions and properties are significant concerns. ECM-based products such as porcine small intestine submucosa (SIS) and fish skin have also gained popularity in clinical studies and are briefly described below (57,97).

Chitosan in combination with marine peptides extracted from Tilapia exhibited significant antibacterial activities (average inhibition circle diameters were 2 mm and 2.5 mm for E. *coli* and 3 mm and 4 mm for S. *aureus*) and improved cell migration and skin regeneration through up-regulation of FGF2 and VEGF (*$p < 0.05$ and **$p < 0.01$) (101).

A bilayer wound dressing composed of SIS membrane with SIS cryogel enhanced wound healing process in rat model (88). The SIS membrane (upper layer) reduced bacterial infection and maintained a moist environment at the wound. The SIS cryogel (bottom layer) was responsible for promoting keratinocyte proliferation and migration. SIS bilayers demonstrated an average amount of wound healing of 90.63%, higher than control, commercial gelatin sponge and both SIS membrane and SIS cryogel alone (47.96%, 67.27%, 79.83%, 86.98% respectively) (102).

PU, PEG and PLGA are three of the most widely used synthetic biopolymers (Table 5). Despite their biocompatibility issues, synthetic biomaterials have a key advantage as they can be synthesized and modified in a controlled manner according to the specific requirements (97).

Table 5: Properties of synthetic polymers for wound healing application (96,99).

Synthetic polymer	Properties
Poly(ε-caprolactone) (PCL)	Biocompatible, biodegradable, non-toxic, bioresorbable and has water retention capacities. Fibrous structure similar to ECM architecture. Limited by its poor antimicrobial properties when used alone.
Polyurethane (PU)	Its semi-permeability which protects the wound from infection and provides a moist environment. Drainage properties that decrease the risk of swelling. Adherence is a limitation.
Polyethylene glycol (PEG)	Hydrophilic, biocompatible, non-immunogenic, flexible and compatible qualities. Growth factors have higher affinity for PEG. Adhesiveness might damage granulation tissue. Composite materials are needed in order to incorporate antibiotics and other drugs.
Poly(lactide-c-glycolide) (PLGA)	Biocompatible, good mechanical strength, and its ease of manipulation in desirable shapes and sizes.

Table 6: Examples of wound dressings commercially available (67,68).

Type	Constituent	Examples
Films	Polyurethane	Tegaderm, Blisterfilm, ClearSite, Comfeel film, Suresite, Procyte, OpSite, Bioclusive, Dermaview..
Foams	Polyurethane	PolyMem, COPA, Optifoam, Gentleheal, Allevyn, Tielle, Flexzan, Biopatch, Biatain, Cutinova, Ivalon, Reston.
Hydrogels	Glycerin	Biolex, Curasol gel, Elasto-Gel, flexigel, IntraSite gel, Restore Gel, Hypergel, tenderwet, SoloSite, Vigilon.
Wafers	Hydrocolloids	DuoDERM, Restore plus, Granuflex, Comfeel, RepliCare, Exuderm, Tegasorb, DuoFilm, Cutinova Hydro, Nuderm, MediHoney.
Hydrogels	Alginate	Calcicare, SeaSorb, Sorbsan, Kaltostat, Maxorb, AlgiSite, AlginSan, Algivon, MediHoney, Tegagen, PolyMem, Algidex Ag, Algivon.

6.2 New advances including Nanotechnology

An increasing number of innovative nano-based therapies have emerged in the field of wound healing. Micelles, polymeric NPs, nanoemulsions, liposomes, among others have been used to improve wound healing at different stages. In this case, the drug itself may be formulated at a nanoscale such that it can function as its own 'carrier' or nanomaterials may be used as drug delivery vehicles. Some studies have been conducted to explore the ability of these systems for wound healing applications (69). Figure 11 schematically represents the different nano-based approaches investigated for wound healing application in the different stages of healing process.

6.2.1 Organic Nanomaterials

6.2.1.1 Micelles

Micelles have a hydrophobic inner core surrounded by hydrophilic shell in aqueous solution, making them an ideal deliver candidate for both hydrophilic and hydrophobic agents. Polymeric micelles present good colloidal stability, greater cargo capacity, biocompatibility, non-toxicity, and controlled drug release (69).

Curcumin-loaded micelles with associated PEG-PCL hydrogel exhibited higher collagen content (22.4 ± 2.2 μg/mg) than curcumin-loaded micelles (Cur-M) (16.9 ± 2.3 μg/mg), blank micelles in hydrogel (M-H) (13.3 ± 1.9 μg/mg) and control group (11.3 ± 1.8 μg/mg) in both linear incision and full-thickness excision wound model in rats, also showing a significantly higher granulation (4.17 ± 0.41) and wound maturity scores (4.00 ± 0.63) *versus* Cur-M (3.33 ± 0.52 and 3.00 ± 0.63, respectively), blank M-H (2.33 ± 0.52 and 2.17 ± 0.75, respectively) or control groups (2.17 ± 0.41 and 2.00 ± 0.63, respectively) (103).

Another example was based on the encapsulation of silver sulfadiazine in chitosan oleate micelles. In this case, this association proved not only to increase drug concentrations, surface to volume ratio and dispersion but also to protect human cells against the drug's cytotoxic effects without affecting its antimicrobial properties. The results confirmed that the silver sulfadiazine micelles improved antimicrobial activity on S. *aureus* and E. *coli* strains (with MIC values of a 50 μg/ mL and 25.8 50 μg/ mL, respectively) compared to the control suspension (150 50 μg/ mL for S. *aureus* and 37.5 50 μg/ mL E. *coli*) (104).

Recently, an *in vitro* study in diabetic rats evaluated the anti-diabetic and wound healing effects of Cur-loaded mixed polymeric micelles based on chitosan, alginate, maltodextrin, Pluronic F127, Pluronic P123, and Tween 80. It was concluded that the developed formulations with highest amounts of Cur (higher than 48.74 ppm) could accelerate the wound healing response showing visible improvement in wound closure on the 14th day and reducing the elevated blood glucose level and lipid profile, clearly demonstrating its potential as diabetes-controlling and wound healing agent (105).

Another promising example is PluroGel which is based on a micelle matrix technology, made of a cell-friendly surfactant, and its claimed to maintain moisture in the wound and control fluid loss, helping to protect the wound and permitting a less painful removal (69).

6.2.1.2 Polymeric NPs

Polymeric NPs are made of biodegradable polymers or copolymers, in which the drug can be dissolved, entrapped, encapsulated or attached. They can be composed of natural, synthetic, and semisynthetic polymers, like gelatin, albumin, alginate, chitosan, poly(glycolic acid) and their copolymers, PLGA, PCL, poly alkyl-cyanoacrylate, etc. They have the advantages of controlled/sustained release, high encapsulation degree, improved bioavailability, and biocompatibility with tissues and cells (69,106).

In terms of polymeric NPs in wound healing, several examples are described in literature. As example, in full-thickness excisional model, the treated mice with host defense peptide (LL37) and lactate encapsulated in PLGA NPs exhibited higher granulation tissue formation (LL37 activates epidermal cells and fibroblasts by up-regulating IL-6), greater re-epithelialized composition and collagen content and enhanced angiogenesis (induced by VEGFα production through up-regulated IL-6) due to the combined effects of LL37 and lactate. In addition, PLGA-LL37 NP also modulated the inflammatory wound response through down-regulation of TNFα expression in mouse macrophage cells (107).

In a murine burn model, a silane-hydrogel nanoparticle vehicle utilized to incorporate Amphotericin B resulted in equivalent or enhanced killing efficacy against *Candida spp.* with 72.4–91.1% reduction in comparison to untreated control and traditional formulation groups. Furthermore, the wounds treated with the Amphotericin B nanoparticles demonstrated significant reduction of fungal biofilm metabolic activity, ranging from 80 to 95% (108).

6.2.1.3 Dendrimers

Dendrimers are synthesized from branched monomers that emerge radially from the central core (109).

The release efficacy of minicircle plasmid DNA encoding VEGF combined with arginine (Arg)-grafted cationic dendrimer was evaluated on wounds of diabetic mice (110). The results confirmed that the injection of the polycomplex resulted in rapid proliferating basal cells and abundant collagen deposition and helped wounds heal faster than the ones treated with the naked VEGF plasmid.

Gelatin scaffolds with poly(amidoamine) (PAMAM) demonstrated relatively higher cellular adhesion and proliferation of both keratinocytes and fibroblasts associated with increased gene expression of native collagen type I of fibroblasts (111). Furthermore, the expression of angiogenesis stimulators such as HIF1α and VEGF were also higher in PAMAM blended gelatin matrix.

6.2.1.4 Nanoemulsions

Nanoemulsions are colloidal systems consisting of emulsified oil and water systems, where the core of the particle is either oil or water and can be used as carriers of poorly water-soluble drugs. Currently, nanoemulsions are mainly found in cosmetic applications (69). High drug loading ability, enhanced drug solubility and bioavailability, relatively easy preparation and scale-up, controlled drug release, and protection from enzymatic degradation are the major advantages of nanoemulsions as drug delivery carriers (69,112).

A phenytoin-loaded alkyd nanoemulsion producing phenytoin concentrations of 25 and 50 mg/mL showed an enhancement in closure activity after 36 h producing 75%-82% “scratch area” compared with phenytoin solutions of equivalent concentration and achieved a significantly higher controlled-release property that maintained the optimal phenytoin level for growth of keratinocyte cells (113).

In another research, it was evaluated the wound healing effects of nanoemulsion formulations of *Eucalyptus* essential oil (EEO) in comparison with pure EEO and standard gentamycin. The optimized nanoemulsion formulation showed comparable results with standard gentamycin-treated animals but presented significant enhancement in collagen content as compared with pure EEO and negative control. The EEO nanoemulsion significantly improved the wound contraction from day 12 to 24 as compared with control （$p < 0.05$) (114).

6.2.1.5 Liposomes

Liposomes are nanosized vesicular structures consisting of an internal aqueous compartment surrounded with phospholipid bilayers. Liposomes offer many advantages; they are safe, biodegradable, non-toxic, biocompatible, and can encapsulate both water-soluble and lipophilic substances (98). The most common disadvantages of liposomes arise partly from poor stability due to potential phospholipids oxidation and hydrolysis as also leakage and fusion of encapsulated drug/ molecules (115).

A bFGF-loaded liposome with silk fibroin hydrogel core showed to improve the stability of bFGF in wound fluids and accelerate the wound closure in mice through the promotion of granulation tissue formation, collagen deposition, angiogenesis, and re-epithelialization (116).

In a different study, authors reported the benefits of topical application of Cur liposome formulation compared with the drug alone. The experiments conducted in rats showed that the Cur -loaded liposomes were capable of improving closure rate as seen on 14^{th} post-wound day ($93.67 \pm 3.56\%$, $p < 0.05$) (98).

In a recent report, a dual deformable liposomal ointment containing retinoic acid (TRA) deformable liposomes and EGF cationic deformable liposomes exerted a synergistic effect leading to facilitated cell proliferation and migration, stimulated wound closure, promoted skin appendage formation and increased collagen production. Furthermore, TRA showed to up-regulate the expression of EGF receptor and HB-EGF suggesting that the synergistic effect created by TRA and EGF could result from the increased expression of these factors (117).

6.2.1.6 Cyclodextrins

Cyclodextrins (CDs) are a family of cyclic oligosaccharides produced from starch by enzymatic conversion containing glucopyranose units with a hydrophilic outer surface and a lipophilic central cavity. CDs have mainly been used as complexing agents to increase aqueous solubility, bioavailability, and stability of poorly water-soluble drugs (118).

Hydroxypropy-beta-cyclodextrin complexed with insulin allowed a more constant and prolonged drug a release and thus improvement of the re-epithelialization process, matrix remodeling, neovascularization, and inflammatory response modulation (119).

CD-modified sacran hydrogel films also confirmed the potential of these systems for wound healing applications by improving wound closure, probably due to its higher moisturizing properties compared to those of the sacran-hydrogel films without CDs. Results showed that sacran, a novel megamolecular polysaccharide, could enhance the skin barrier function during the wound healing process and mediate the production of cytokines (such as IL-5 and TNF-α) and thus, influence the anti-inflammatory activity (120).

6.2.1.7 Other Lipid-based NPs

Solid lipid NPs (SLNs) and nanostructured lipid carriers (NLCs) were developed as potential alternatives (106).

A previous work described the preparation of NLCs with phenytoin. The aim of this study was to achieve a better entrapment efficiency and sustained release on diabetic foot ulcer (DFU) healing (121). The results clearly demonstrated that the wound area was reduced with phenytoin-NLC hydrogel (0.26±0.22 cm^2) compared to blank (7.24±3.65 cm^2) and PHT-hydrogels (2.05±0.89 cm^2).

In another study, it was reported the clinical potential of topical rosemary essential oil (REO)-loaded into the NLCs for the treatment of infected wounds. REO-NLCs-treated group exhibited an enhanced antibacterial activity against *S. epidermidis, S. aureus, L. monocytogenes, E. coli* and *P. aeruginosa* and MIC values ranged between 5.34 ± 1.27 and 10.13 ± 1.67 mg/mL. In addition, it simultaneously incremented IL-3, IL-10 and VEGF levels ($p < 0.001$), neovascularization ($p < 0.05$), fibroblast infiltration, collagen deposition and re- epithelialization (122).

Equally, *in-vitro* and *in-vivo* evaluations were carried out to evaluate the use of semi-solid formulations containing Cur and ampicillin SLNs as burn wound healing agents. Making the most of the synergistic effect of ampicillin and Cur, the two semi-solid preparations simultaneously increased the wound healing rate and had reasonable anti-bacterial effects (123).

Using diabetic wounded mice models, whereas overproduction of TNF-α has an essential role in the abnormal and under regulated wound healing process, researchers investigate the potential of siRNA-loaded lipid NPs to reduce chronic inflammation and facilitate wound closure. Topical application of NPs resulted in approximately 50% TNF-α gene silencing, almost reaching typical baseline levels of normoglycemic mice and, thus treated wounds showed wound area reduction and faster healing closing within 13 days (124).

6.2.2 Inorganic Nanomaterials

Researchers have explored various kinds of approaches for the synthesis of INPs including physical and chemical methods like laser ablation, pyrolysis, lithography, chemical vapor deposition, sol–gel techniques and electro-deposition as listed in Table 7, which are very expensive and hazardous. In an attempt to develop a low-cost, eco-friendly and energy-efficient approach, researchers have exploited the potential of much greener methods using microbial systems, plant systems, etc. (63,125).

Table 7: Different synthesis approaches of inorganic NPs (60).

Physical approach	**Chemical approach**	**Biological approach**
Evaporation-condensation	UV-initiated photoreduction	Plant sources: Leaves, Root, Fruit, Bark Phytochemicals
Laser ablation	Sono-electrochemistry	Microbial sources:Bacteria, Fungus, Algae, Virus
Thermolysis	Microemulsion techniques	Marine sources
Spluttering	Electrochemical synthetic method	
	Irradiation methods (Microwave-assisted synthesis and γ-ray irradiation)	
	Sol-gel method	
	Hydrothermal synthesis	

Silver, zinc oxide, gold, silica, graphene, copper, cerium oxide, terbium oxide, titanium dioxide and other materials have demonstrated their wound-healing properties using *in vitro* and *in vivo* models which are summarized in this work.

The use of silver-containing agents has been practically limited to topical silver sulfadiazine (SSD) for burn wound management due to their toxicity associated with higher doses. A renewed interest in silver was rekindled after the development of strategies such as nano-crystalline silver or silver NPs that are effective at a very low concentration because of their high surface to volume ratio which minimizes the chance for tissue toxicity compared to the conventional silver (including SSD and silver nitrate) (63,126). Besides, with the increasing development of multidrug-resistant bacterial strains, the use of silver NPs (AgNPs) proved to be a promising alternative to antibiotics either by their ability to kill bacteria through numerous mechanisms of action or by their function as drug carriers. Although these mechanisms are still unclear, there are several accepted suggestions. The exposure of silver ions to bacterial cells can lead to damage to the membrane and all the cellular components leading to apoptosis. The production of ROS has also been shown to

influence this mechanism of cell destruction. Further, the production of ROS reported to inhibiting ATP synthesis, which, in turn, causes DNA damage (63).

Many studies on AgNPs have focused on burns, but diabetic ulcers have also been studied (61). Along with their powerful antibacterial properties, AgNPs showed to be able to promote wound healing via immune regulation. The use of hybrid scaffolds made of metallic nanosilver particles-collagen / chitosan in a rat model showed to up-regulate the macrophages activation and fibroblast differentiation into myofibroblast through accelerated migration of fibroblasts and increased expression of α-SMA (127). Furthermore, the decreased level of collagen in fibroblasts treated with AgNPs suggest that they could be useful in preventing and perhaps treating keloids and scars (128). In another report, authors found that nano-silver-decorated microfibrous eggshell membrane could trigger re-epithelialization, granulation tissue formation and wound healing via enhancing cell proliferation and controlling inflammation response (129). Despite this, the underlying mechanism by which silver interacts at the molecular level is not yet precise.

Green AgNPs were synthesized using the *Calliandra haematocephala* leaf extract containing contains gallic acid which was responsible for the reduction of silver nitrate to silver NPs. The bio-synthesized NPs displayed significant antibacterial activity against E. *coli* (130). Results from animal models on burned skin confirmed that animals treated with AgNPs exhibit significantly faster wound closure than those treated with SSD (128).

Acticoat® and PolyMem silver are examples of commercially available wound dressing containing AgNPs, both composed of nanocrystalline silver (131). However, the application of these conventional formulations is limited due to safety concerns. Despite several efforts, no other inorganic NPs have got approval. Then, we have some other inorganic based nanomedicines which are under clinical investigations.

Zinc oxide (ZnO) NPs have also been promising due to their effective cell penetration, immunomodulation and antimicrobial capacity *in intro* e *in vivo* experiments (132). Studies in mice proved that ZnO particles are very effective in preventing wound infection; thus, they are currently being investigated for antimicrobial wound dressings, showing to be less toxic to mammalian cells than silver (61,133,134). For example, studies have proven the effectiveness of ZnO NPs in the prevention of microbial biofilms formation by P. *aeruginosa*. The main bactericidal mechanism of ZnO NPs seems to be related to the formation and release of ROS. However, the generation of ROS also plays a crucial role in ZnO NPs -induced cytotoxicity (61,63). In a clinical report was evaluated the ZnO NPs -induced cytotoxicity on sheep fibroblast cell culture. The authors concluded that

nanoformulation of ZnO NPs using sodium alginate and gum acacia exhibit a healing effect at a low concentration minimizing cytotoxicity due to decreased ROS generation in fibroblast cells (135). The incorporation of ZnO NPs into heparinized poly(vinyl alcohol) /chitosan/hydrogels were reported to enhance antibacterial activity more than 70% against E. *coli* and S. *aureus* bacteria compared to sample without ZnO. Furthermore, results of cell viability performed on mouse fibroblast cells showed no toxicity within 24 to 48 h and, therefore, the produced hydrogel samples are completely biocompatible (136).

Although AgNPs and ZnO NPs are the most investigated for advanced technologies, other NPs are being considered as potential therapeutic options (61,137).

Studies in diabetic mice have shown that gold (Au) NPs co-delivered with epigallocatechin gallate and α-lipoic acid accelerated wound healing up-regulating angiogenesis and down-regulating the expression of the receptor for advanced glycation end-products (which is known to increase ROS formation) (138). On the other hand, nanocomposite collagen scaffolds incorporating AuNPs showed improvement on the wound closure while significantly suppress the inflammatory response and enhance re-epithelialization, neovascularization and granulation tissue formation (139).

In a recent study, PEGylated and cationic charged gold nanorods demonstrated a remarkable wound healing efficiency after 14 days. Both hydrogels were capable of promoting skin re-epithelialization and collagen deposition and regulate gene expression of several inflammatory mediators. Besides, they had strong bactericidal effects demonstrating 2.7-log 2 reduction (99.8%) and 4-log reduction (99.99%) of viable bacterial count against *S. aureus* and *P. aeruginosa*, respectively (140). It is known that Au NPs exert antibacterial activity predominantly in two manners: (1) by changing the membrane potential and inhibiting enzyme ATP synthase, thus reducing the metabolism process, (2) by preventing the combination of a ribosomal subunit with tRNA (141).

Magnetic NPs such as magnetite (Fe_3O_4) have been also used due their distinctive properties that are susceptible to a magnetic field (61). A study was performed to investigate the influence of a nano-coated wound dressing containing magnetite NPs and *Satureja hortensis* essential oil on *C. albicans* colonization rate and biofilm formation. The results demonstrated that the modified wound dressing strongly inhibited fungal adherence and biofilm formation (85). Similarly, a wound dressing with nano-coating based on Fe_3O_4 NPs and patchouli essential oil showed an efficient inhibition of S. *aureus* biofilm formation. EO have shown to have bactericidal effects through multiple mechanisms of

action and targets, offering an alternative to antibiotics and decreasing the probability of bacterial resistance (142).

Studies have been made to investigate the controlled release of nitric oxide (NO) because of its important role in inflammation, cell proliferation, ECM deposition, angiogenesis, and matrix remodeling besides its antimicrobial properties (134). NO-releasing silica (SiO_2) NPs demonstrated a very high kill rate against P. *aeruginosa* and E. *coli* (with ≥ 5 logs of killing for both gram-negative species), intermediate efficacy against C. *albicans* (3 logs of biofilm killing) and lower efficacy against gram-positive S. *aureus* and S. *epidermidis* biofilms (~2 logs of biofilm bacteria). (143). Additionally, studies have reported the effects of silica NPs as sources of silicic acid on the proliferation and migration of fibroblasts. Authors suggested that silica NPs (positively charged) were easily internalized by fibroblast, which led them to release silicic acid intracellularly, promoting cell migration and ultimately wound healing (144). Also, researchers showed promising results from hydrophilic chitosan–silica hybrid sponge which was able to promote not only fibroblast proliferation and migration and endothelial cell proliferation but also the secretion of growth factors such as TGF-β, revealing enhanced collagen deposition and angiogenesis in a porcine model (145). In a murine model, a PVP (polyvinylpyrrolidone) gel of nano-structured SiO_2 led to improved cell proliferation and cell migration (due to moisture properties), advanced degree of re-epithelialization after 9 days, and moderately improved neo-angiogenesis (652.9 ± 142.6 vessels/mm^2 *versus* 489.3 ± 207 vessels/mm^2 of the control group) (146).

Graphene and their derivatives have also attracted considerable interest as wound healing materials. Cur incorporated in collagen functionalized nano graphene oxide scaffolds restricted the bacterial growth of gram-negative bacteria, namely P. *aeruginosa* and gram-positive bacteria, namely S. *aureus* (147). Incorporation of reduced graphene oxide (GO) in isabgol (a natural carbohydrate polymer derived from psyllium husk) solution proved to be promising in healing wound on diabetic wounds. These nanocomposite scaffolds exhibited faster re-epithelialization, higher shrinkage temperature of the granulation tissues (due to the deposition of crosslinked collagen), increased collagen concentration, complete wound closure on day 16, and increased angiogenesis in treated Wistar rats (148). Likewise, a hybrid GO/Ag/Arg nanocomposite embedded in electrospun PCL scaffold demonstrated not only a tremendous antibacterial effect on both against E. *coli* and S. *aureus* bacterial species (MIC of 0.02 and 0.05 μg/mL, respectively) but also angiogenesis properties by controlling ROS formation (149).

Studies suggest that VEGF expression is sensitive to copper; thus, copper plays a vital role in wound healing by inducing angiogenesis. However, the toxicity of copper salts or oxides is raising concern. The incorporation of folic acid was proven to delay the release of copper ions, which reduce cytotoxicity and enhance cell migration. The stabilized system promoted angiogenesis, collagen deposition and re-epithelialization, and increased wound closure rates (63,150). Copper oxide NPs were synthesized by using *Ficus religiosa* leaf extract. The authors revealed that the nanocomposites exhibited enhanced wound healing efficacy in rats by inhibited pathogenic bacteria such as K. *pneumoniae*, Shigella *dysenteriae*, S. *aureus*, Salmonella *typhimurium* and E. *coli*. The copper oxide NPs treated wounds showed 93% of closure compared to 80% of the control group with massive formation of macrophages and fibroblasts, a higher amount of collagen fibers and a greater degree of new blood capillary formation (151).

Topical application of cerium dioxide NPs (CNPs or Nanoceria) to treat neuropathic DFUs demonstrated a greater efficiency in the prevention of secondary infections and reduction of oxidative damage due to their bacteriostatic activity and anti-inflammatory properties (152). The ability of CNPs to penetrate the wound tissue and reduce oxidative damage had already been proven using mice model, showing a massive promise for wound healing applications. Moreover, CNPs enhances the proliferation and migration of fibroblasts, keratinocytes and vascular endothelial cells (153). MicroRNAs have been identified as critical regulators in the production of pro-inflammatory cytokines at the post-transcription level. In particular, miR-146a has been described to have decreased expression in diabetic wounds, being responsible for the increased gene expression of the pro-inflammatory cytokines IL-6 and IL-8/MIP-2. Authors used CNPs and conjugated them with miR-146a to target ROS and inflammation. The treatment with CNPmiR146a conjugate demonstrated accelerating wound healing in both murine and porcine model. CNP-miR146a show great potential for wound healing applications using a combination of ROS scavenging properties of CNPs and anti-inflammatory properties of miR-146a (154).

Terbium oxide (Tb_4O_7) NPs demonstrated their bactericidal effects and wound healing properties in wound infection mouse model. The results of the study showed that their oxidase- and peroxidase-like activities allow these NPs to consume antioxidant biomolecules and generate ROS within bacteria cells. These findings proved great effectiveness of Tb_4O_7 NPs as antibacterial agents (155). It has also been showed the wound healing efficacy of terbium hydroxide nanorods (THNRs) which demonstrated excellent pro-angiogenic properties due to reported evidence of enhanced viability, proliferation and

migration of endothelial cells. This was evidenced by various cell-based *in vitro* assays (63). A chick embryo *in vivo* model reported that THNRs promote wound healing by ROS in a NOX-dependent manner which further activates PI3K/Akt/MAPK signaling cascade and intracellular formation of NO, ultimately inducing angiogenesis in a downstream signaling manner. Likewise, the application of these nanorods in punch biopsy mouse model demonstrated accelerated wound closure in THNRs treated mice compared to untreated and vehicle control groups. Moreover, there was no significant elevation of serum concentrations between THNRs administered and untreated mice groups, validating their non-immunogenicity nature (156). In another work, nanorods consisting of europium (Eu) hydroxide and terbium (Tb) nitrate were developed to further investigate the nanoparticle-enhanced angiogenesis *in vitr*o, using zebrafish embryonic primary cell culture and *in vivo*, using transgenic zebra fish models. The results demonstrated that Eu and Tb NPs could generate reactive oxygen species H_2O_2 and subsequently provide an *in vivo* and *in vitro* pro-angiogenesis effects (157).

The use of titanium dioxide in NPs has also been promising as wound healing enhancer as well as an antibacterial agent against Gram-positive and Gram-negative bacteria. For example, the wound healing potential of bacterial cellulose and titanium dioxide (TiO_2) nanocomposites (BC–TiO_2) was evaluated in burn wound model. The nanocomposites dressings showed remarkable wound healing activity through re-epithelialization, fibroblast migration and angiogenesis process and displayed 81 ± 0.4% and 83 ± 0% inhibition against E. *coli* and S. *aureus*, respectively (158). Bio-synthesized TiO2 NPs by using *Moringa oleifera* Lam. leaf extract were capable of enhancing wound healing activity in Albino rats showing 92.36% of healing. TiO_2 NPs have also been obtained from *Morinda citrifolia* L. extract, *Trigonella foenum-graecum* extract, *Curcuma longa* extract, *Aloe Vera* extract, and others, all showing significant antibacterial efficacy. Bacterial and fungus extracts have also been considered for the thesis of TiO2 NPs, being the use of fungi more advantageous as they offer certain benefits such easier scaling up and extraction, large surface area and economic viability. Some examples of these microbial species are *Aeromonas hydrophila*, *Bacillus subtilis*, *Lactobacillus*, *Aspergillus* species including A. *niger*, A. *flavus*, A. *tubingensis*, *Fusarium oxysporum*, *Saccharomyces Cerevisiae*, among others (63).

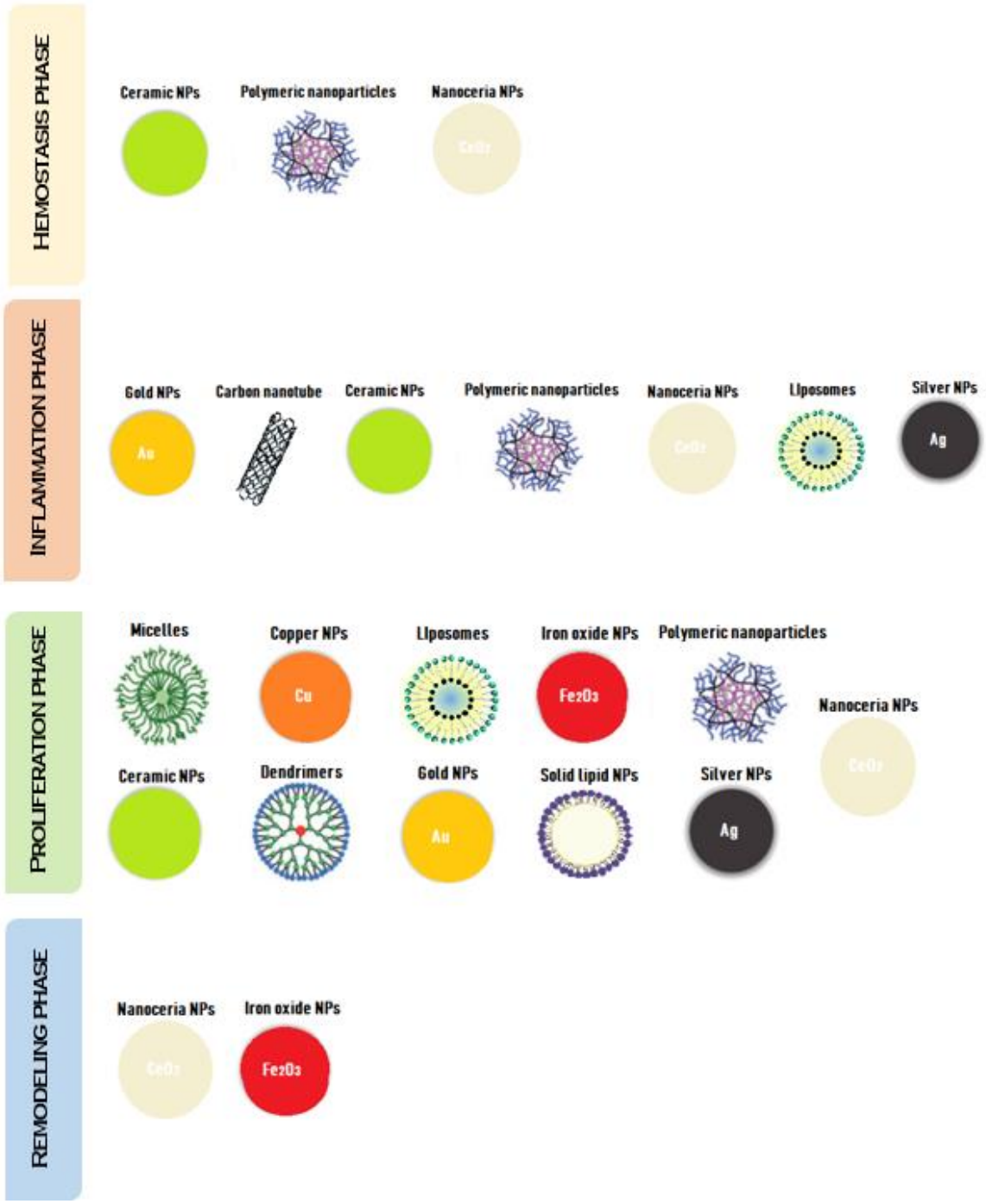


Figure 11: Schematic representation of the nanotechnology-based therapies employed in wound healing.

6.3 Topical application of autologous products

6.3.1 Platelet-rich plasma

Platelet-rich plasma (PRP) can be defined as an autologous blood product containing an increased dose of growth factors due to its higher concentrations of platelets. PRP has shown satisfactory results in the treatment of acute cutaneous wounds, chronic skin ulcers and burns. PRP is a simple, safe and cost-effective method, as it can be easily obtained from the patient's own blood after a centrifugation process, through which it is possible to control the dose of GFs and proteins that are released upon activation (53,75). The release of multiple growth factors and differentiation of factors upon platelet activation are the significant advantages of PRP over the use of single recombinant human growth factor delivery (159).

The research team of Biotechnology Institute has developed the PRGF-Endoret® which received European certifications (European CE mark), with application in several therapeutic areas (oral surgery, dermatology, locomotor system and ophthalmology) (160).

The use of PRP reported in human clinical trials is mainly related to chronic conditions, such as diabetic ulcers. A study on 24 patients with non-healing ulcers treated with subcutaneous PRP injections together with topical application of autologous PRP gel demonstrated a reduction in pain and improvement of quality of life, with an evident decrease of the wound size (159). Similar positive results were obtained in other clinical trial showing a reduction in area, volume and undermining of the ulcer on 63 of 65 non-healing ulcers (161).

As shown, several studies have been conducted on the use of PRP revealing promising results; however, its beneficial effects in clinical procedures are not entirely clear due to the limitedness of critical scientific data (159).

6.4 Engineered skin substitutes

Skin substitutes are coverage materials that aid in wound closure and are designed to replace the functions of the skin, either temporarily or permanently (162). The currently available skin substitutes are time consuming because of their extensive procedures and production time, bear a risk of infectious agent transmission, have insufficient vascularization, and are costly. In the future, technologies like bioprinting can automate

tissue engineering and allow higher reproducibility, better safety, larger-scale production, and higher efficacy (163).

6.4.1 Biological Skin Substitutes

Xenografts, allografts, and amnion are usually used as biological substitutes and they have some advantages over synthetic substitutes as listed in Table 8. Xenografts (especially porcine skin) are mainly used for the temporary coverage of partial-thickness burns. However, these natural constructs can exhibit a likelihood of rejection and infection. The cadaveric skin allograft is the most widely used skin substitute for burn wound care. The amnion is a thin semi-transparent tissue found in the innermost layer of the fetal membrane and can be classified as one of the most effective substitutes to be used in healing or covering partial thickness burn wounds (164).

Table 8: Advantages and disadvantages of biological and synthetic skin substitutes (165).

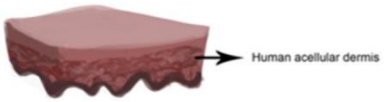
Biological skin substitutes	**Advantages**	More intact extracellular matrix structure which may allow the construction of a more natural new dermis and excellent re-epithelialization characteristics due to the presence of a basement membrane.
	Disadvantages	Can exhibit problems with slow vascularization of the material.
Synthetic skin substitutes	**Advantages**	Can be made on demand with specific characteristics in each case. Therefore, these products can integrate various additives such as growth factors and matrix components to enhance the wound healing process. They could also avoid complications due to potential disease transmission
	Disadvantages	Synthetic skin substitutes generally lack basement membrane and their architecture does not resemble native skin.

6.4.2 Synthetic skin substitutes

6.4.2.1 Dermal substitutes

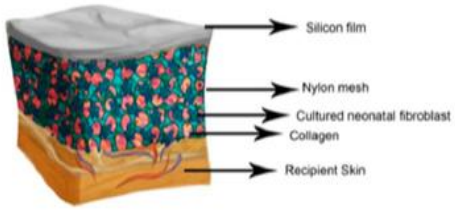
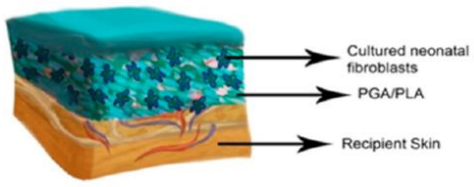
Acellular dermal products, such as Alloderm®, are originate from de-epithelialized cadaveric skin. Alloderm® has been successfully used in partial- and full-thickness burn wounds (162). The characteristics of this acellular dermal substitute are mentioned in Table 9.

Table 9: Acellular Allogeneic Dermal Substitutes.

Acellular Allogeneic Dermal Substitute	Duration of cover	Advantages	Disadvantages	Refs.
 **Alloderm**	Permanent	Acellular and immunologically inert, provides immediate permanent wound coverage and allows the use of thinner autograft as one-stage procedure, 2-year shelf life.	Risk of transmitting infectious diseases, collection fluid risk (seroma, hematoma), high cost, and possibility of donor rejection and requires two procedures.	(162,166)

A silicon-coated nylon-collagen scaffold seeded with fibroblasts from neonatal foreskin has been used as a non-living, temporary, double-layered covering for excised burns, commercially available as TransCyte®. As another example of cellular allogenic skin substitutes, Dermagraft® has been also reported for burn wounds and diabetic ulcers treatment, generated by the culture of living neonatal dermal fibroblasts onto a bioabsorbable polyglactin mesh scaffold (162,163,167). These cellular allogeneic dermal substitutes are presented in Table 10. Other substitutes in this category include ICX-SKN composed of fibrin matrix seeded with neonatal human fibroblasts and Grafix, comprised of cryopreserved amniotic membrane (163,167).

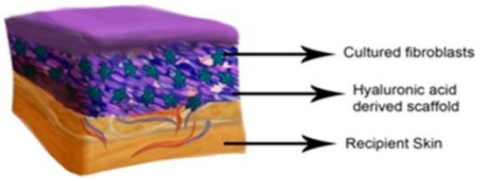
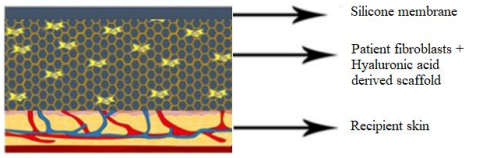
Table 10: Cellular Allogeneic Dermal Substitutes.

Cellular Allogeneic Dermal Substitute	Duration of cover	Advantages	Disadvantages	Refs.
 **TransCyte (DermagraftTC)**	Temporary	Immediate availability, 1.5-year shelf life, ease of storage and direct visual monitoring of the wound bed (due to transparency).	Expensive	(162,166)
 **Dermagraft**	Temporary or permanent	Good resistance to tearing, ease of handling, and lack of rejection.	Poor ECM structure, infections and high cost, used for temporary coverage and 6-month shelf life.	(162,168,169)

Hyalomatrix PA® and Hyalograft 3D® are both autologous dermal substitutes based on HA derivate (Table 11). Clinically, Hyalograft-3D® is primarily used for feet ulcer treatment in combination with epidermal substitute Laserskin®. Autologous cultured

fibroblasts seeded onto a 3D HA derived scaffold are used in Hyalograft 3D® production. Hyalomatrix PA®, a bilayer, esterified HA (Hyaff) matrix or scaffold covered by a temporary silicon layer has been an important tool in wound closure achievement in venous ulcers (170,171).

Table 11: Cellular Autologous Dermal Substitutes.

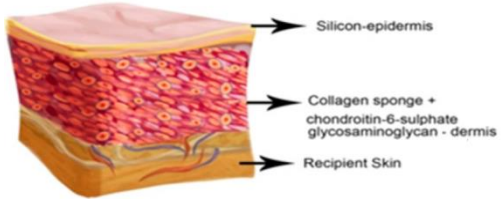
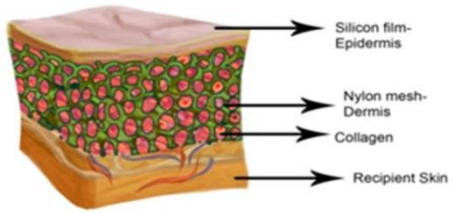
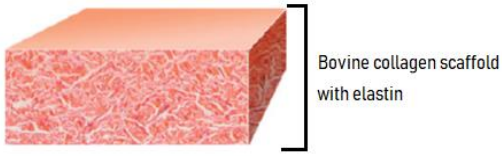
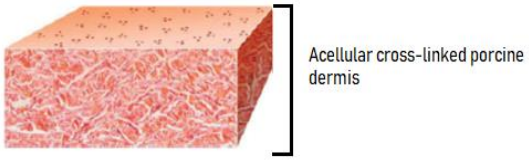
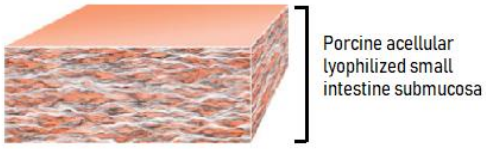
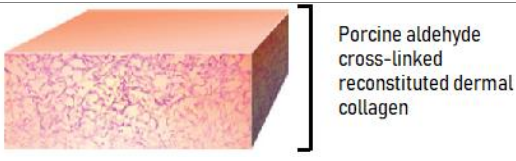
Cellular Autologous Dermal Substitute	Duration of cover	Advantages	Disadvantages	Refs.
 **Hyalograft 3D**	Permanent	Enhanced keratinocyte take, reduced hypertrophy and wound contracture rates when compared with exclusive application of keratinocyte cultures and also contributes towards rapid basement membrane formation.	No pseudo epidermal layer.	(172)
 **Hyalomatrix PA**	Semi-permanent	Off-the-shelf availability, user-friendly application technique, it contributes to a fast pain reduction and improves the re-epithelialization speed with a regenerative mechanism.	Two stage procedure.	(170)

Decellularized dermal products like MatriDerm® and Permacol® are similar to AlloDerm but of animal origin. MatriDerm® is a structurally intact matrix of bovine type I collagen with elastin, whereas Permacol is made of porcine acellular diisocyanite cross-linked dermis (171,172).

Integra® is an acellular scaffold composed of a silicon layer on top of a porous matrix comprising a chemically cross-linked coprecipitate of bovine collagen I and chondroitin-6-sulfate. It is used for wound reconstruction following trauma, cancer removal, and scar revision of all anatomical sites. It has also widely used for the treatment of burns and scar contractures (167).

Biobrane® consists of a nylon mesh cross-linked with porcine dermal collagen and a silicon membrane used in partial-thickness burns (168,171). These xenogeneic acellular matrices are reported in Table 12. Other porcine-derived acellular substitutes like Ez Derm™ and OASIS Wound Matrix are used in chronic and burn wounds treatment (163). Karoderm, SureDerm and GraftJacket are also representatives of acellular dermal matrix products (96).

Table 12: Acellular Xenogeneic Dermal Substitutes.

Acellular Xenogeneic Dermal Substitute	Duration of cover	Advantages	Disadvantages	Refs.
 **Integra**	Semi-permanent	Immediate availability, allowing time for the neo-dermis formation, and good aesthetic results.	Needs a two-step operation, expensive, and accumulation of exudate underneath it that may lead to infection.	(168)
 **Biobrane**	Temporary	One-stage procedure, availability, low pain, short hospital admission time and reduced nursing requirements when compared to traditional dressings.	Risk of infection, reported cases of toxic shock syndrome due to accumulation of exudate underneath it and permanent scarring in partial-thickness scald wounds.	(162,168)
 **MatriDerm**	Permanent	One-stage procedure, off-the-self product and it is capable of promote vascularization, improve stability and elasticity of regenerating tissue.	Still weak scientific evidence.	(166)
 **Permacol Surgical Implant**	Permanent	Good esthetic and functional outcome.	Infection, hematomas and seromas.	(169)
 **OASIS Wound Matrix**	Permanent	Immediate availability, long shelf-life.	Not many clinical data.	(169)
 **EZ Derm**	Temporary	Good aesthetic and functional outcome.	Possibility of disease transmission and not many clinical data.	(169)

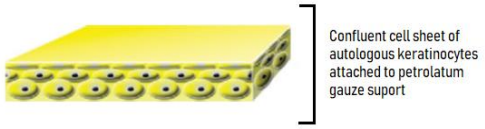
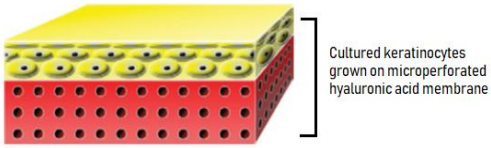
6.4.2.2 Epidermal substitutes

Epicel® is manufactured using autologous keratinocytes attached to petrolatum gauze support and is used for permanent coverage in partial or full-thickness wounds. Laserskin®

is composed of autologous keratinocytes and fibroblasts cultured on the microperforated HA membrane (162). The advantages and disadvantages of both epidermal skin substitutes are indicated in Table 13.

RECELL®, a Spray-On Skin System containing a mixture of keratinocytes, fibroblasts, and melanocytes, obtained from a small sample of the patient's own skin was introduced into clinical practice (173). However, Avita's system has hit a hiccup in their sales; thus, the company has temporarily halted sales in Europe (174). Other marketed autologous epidermal substitutes include EPIBASE, Bioseed-S, MySkin and Epidex (163).

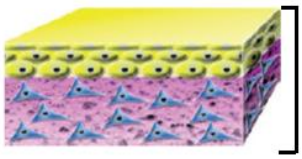
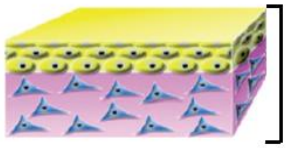
Table 13: Cellular Autologous Epidermal Substitutes.

Cellular Autologous Epidermal Substitute	Duration of cover	Advantages	Disadvantages	Refs.
 **Epicel**	Permanent	Use of autologous cells obviates rejection and permanent large area wound coverage, especially in extensive burns.	3 weeks for graft cultivation, lack of dermal components, 1-day shelf life, expensive and risk of blistering, contractures, and infection.	(162,172)
 **Laserskin®/Vivoderm**	Permanent	Good graft take, low rate of infection and ease of handling during application.	2-day shelf life and expensive.	(162,172)

6.4.2.3 Epidermal/Dermal (Composite)

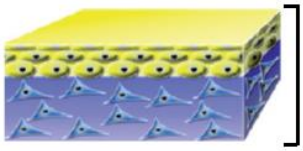
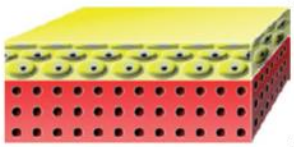
Other commercial skin substitutes such as Apligraf® and OrCel® (Table 14), are constructed from sheets of cells derived from allogenic foreskin (164). OrCel® is a bilayer cellular matrix in which keratinocytes and fibroblasts are cultured into a type I bovine collagen sponge. It has been approved to treat donor sites in burns recessive dystrophic epidermolysis bullosa. Similar to OrCel®, Apligraf® is also a bilayer substitute comprising both cultured human fibroblasts and keratinocytes. It has been used successfully in venous ulcers treatment.

Table 14: Cellular Allogeneic Epidermal/Dermal Skin Substitutes.

Cellular Allogeneic Epidermal/Dermal Skin Substitute	Duration of cover	Advantages	Disadvantages	Refs.
 Bovine collagen I sponge seeded with neonatal foreskin fibroblasts and keratinocytes **Orcel**	Temporary	9-month shelf life, reduced scarring, shorter healing compared to acellular wound dressing.	Cryopreserved, cannot be used in infected wounds, in patients who are allergic to any animal products, or in patients allergic to penicillin, gentamycin, streptomycin, or amphotericin B.	(162,169)
 Bovine collagen I matrix with allogeneic neonatal foreskin fibroblasts and keratinocytes **Apligraf**	Temporary	Enhanced healing after 4 weeks.	Expensive and 5-day shelf life.	(169,172)

Current commercially available autologous dermal/epidermal substitutes include PolyActive® and TissueTech Autograft System (Table 15). Autologous cultured keratinocytes and fibroblasts are seeded into a PolyActive® matrix consisting of polyethylene oxide terephthalate or polybutylene terephthalate. It has been studied as skin and bone tissue regeneration scaffold. The TissueTech Autograft System combines two products for consecutive application: Laserskin® as epidermal and Hyalograft 3D as a dermal replacement. This system is commonly used for DFUs treatment (172).

Table 15: Cellular Autologous Epidermal/Dermal Substitutes.

Cellular Autologous Epidermal/Dermal Skin Substitute	Duration of cover	Advantages	Disadvantages	Refs.
 Cultured keratinocytes and fibroblasts on polyethylene oxide terephthalate (PEO)/ polybutylene terephthalate (PBT) **PolyActive**	Temporary	No immune rejection.	Use of autologous cells may limit the "of-the-shelf" availability.	(169,172)
 Cultured keratinocytes and fibroblasts in the microperforated hyaluronic acid membrane (HAM) **TissueTech Autograft System**	Permanent	May allow definite wound closure.	Requires grafting of two products- not a "true" bilayer skin substitute.	(169,172)

7. Conclusions

Chronic wounds generally cause suffering, increase the susceptibility of infections, decrease the quality of life of patients and in extreme situations, may lead to death. Thus, non-healing chronic wounds and excessive scarring are a tremendous burden for patients and the healthcare system, and for this reason, there is an increased interest in finding more efficient therapies.

Several approaches were described in this thesis. It was clear that the magnitude of wounds as a health care problem is sharply rising. Resources allocated to the research of wounds continue to be disproportionately low and deserves strategic attention.

Given the complexity of the chronic wound pathology and tissue repair process, a better understanding of the wound-healing process is required towards the development of less-invasive and cost-effective treatments. Additional effects have been achieved through the association of different therapeutic approaches. Despite the extensive efforts, there is still a lack of experimental and clinical evidence regarding the use of some technologies in wound care.

Synthetic (PVP, PLGA, PLA, PEG, etc.) and natural (chitosan, cellulose, HA, collagen, etc.) origin polymers are now some of several examples of used materials in the development of new therapeutic approaches. Owing to their biocompatibility, biodegradability and similarity with ECM, the use of natural polymers is prevalent in medicine. Natural antimicrobials agents such as Cur, EO, honey and others have also shown remarkable results in infected wounds. As alternative therapeutic agents, metals such as silver are widely used in clinical practice due to their high potential in treating drug-resistant biofilms. Likewise, nanotechnology has shown promising approaches to prevent drug-resistant biofilm infections in an era where the growing resistance to antibiotics represents a huge concern. Stem cell nanotechnology has also gained considerable attention in the treatment of chronic and acute wounds as well as scar management. BM-MSCs have been the gold standard in clinical trials so far, but UC-MSCs have shown better therapeutic abilities in terms of angiogenesis and cell migration through a painless collection procedure. Stem cells are among the most promising cell sources for tissue regeneration, but their use appears to be limited due to immunogenicity. Key challenges in stem cells therapy which remain to be addressed, is to improve our understanding of therapeutic mechanisms of stem cells (the composition of

the factors secreted, as well as the environmental factors that stimulate their release) and to improve methods of stem cell delivery.

In terms of growth factor and gene therapy, the last shows to be more advantageous since it does not require continuous administration or high doses to exert the desired effects due to the higher stability of DNA. To overcome their low *in vivo* stability, DDSs act as important mechanisms to prevent growth factor degradation at the wound site, allowing sustained release, and ultimately optimizing treatment effectiveness. The identification of novel target genes and gene delivery systems are some of the aspects that need to be addressed and that may help to translate gene therapy into future clinical management of wound healing.

The major drawback of engineering skin substitutes is the lack of other main skin components or main cell types besides keratinocytes and fibroblasts, but research is currently ongoing in this field in order to allow integration of components such as melanocytes, fat, and hair follicles so that functions such as temperature control, immune regulation, insulation and pressure sensation are ensured.

In the near future, new advanced systems like nanoproducts will undoubtedly provide efficient formulations for the treatment of wounds management and skin regeneration, bur deeper understanding of the complex, highly regulated process of wound healing is necessary for the more rational design of such therapies in order to improve patient care and quality of life.

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Annex

Table 16: Growth factors involved in wound healing (17,175–184).

Name	Produced by	Action
Epidermal growth factor	Platelets, macrophages, keratinocytes	Promotes the proliferation and migration of keratinocytes. Stimulates the fibroblast motility, fibronectin synthesis, angiogenesis, fibroplasia and collagenase activity.
Vascular endothelial growth factor	Endothelial cells, keratinocytes, platelets, macrophages, fibroblasts	Promotes angiogenesis during tissue hypoxia under the influence of hypoxia inducible factor (HIF1α). Increases the vascular permeability of blood vessels.
Transforming growth factors (alpha and beta)	Platelets, macrophages, fibroblasts, keratinocytes, T lymphocytes	Recruitment of inflammatory cells, keratinocyte chemotaxis and proliferation, fibroblasts migration. Inhibits production of MMPs and upregulates TIMP, regulating the accumulation of ECM components (collagen, fibronectin, and hyaluronic acid). Promotes angiogenesis
Keratinocyte growth factor (also called fibroblast growth factor-7, FGF-7)	Fibroblasts	Proliferation, migration, and differentiation of keratinocytes.
Insulin-like growth factor	Platelets, fibroblast and other epithelial cells	Re-epithelialization and granulation tissue formation of epidermal tissue.
Hepatocyte Growth Factor	Mesenchymal cells	Epithelial repair, granulation tissue formation and neovascularization; regulation of cell growth, motility and morphogenesis in epithelial and endothelial cells.
Tumor necrosis factor	Endothelial cells, macrophages, keratinocytes and fibroblasts	Proinflammatory, formation of the extracellular matrix (inducing the generation of proteoglycan and fibronectin by fibroblasts).
Fibroblast growth factors acidic and basic	Macrophages, mast cells, T-lymphocytes	Promotes angiogenesis and granulation via endothelial cells and fibroblasts, respectively. Stimulate the migration and proliferation of keratinocytes, thereby improving wound re-epithelialization.
Interleukins	Macrophages, keratinocytes, endothelial cells, lymphocytes, fibroblasts, osteoblasts, basophils, mast cells	IL-1: Induces the migration of inflammatory cells and keratinocytes. IL-6: Has mitogenic and proliferative effects on keratinocytes and is chemoattractant for neutrophils. IL-8: Potent chemoattractant for neutrophils, basophils and T cells, impact on the migration of fibroblasts, keratinocytes and endothelial cells.
Platelet-derived growth factor	Platelets, macrophages, and endothelial cells	Attracts fibroblasts, neutrophils, monocytes and smooth muscle cells to the site of injury. It also promotes the proliferation and migration of endothelial cells, contributing to angiogenesis. Accelerate the production of new extracellular matrix components and subsequent collagen synthesis by fibroblasts. Stimulates fibroblasts to contract collagen matrices and induces the fibroblast to myofibroblast conversion.
Colony-Stimulating Factors	Stromal cells, fibroblasts, endothelial cells, lymphocytes	Granulocyte colony-stimulating factor (G-CSF): Stimulates granulocyte proliferation. Granulocyte Macrophage Colony Stimulating Factor (GM-CSF): Stimulates granulocyte and macrophage proliferation, promotes the migration and differentiation of epithelial cells and keratinocytes.

Table 17: Advantages and challenges of different delivery systems in wound healing dressings (82,92,134,185–189).

	Advantages	Challenges
Stem cells therapy	Multiple differentiation, high frequency, facility of isolation and characterization, capacity to secrete growth factors and cytokines and ability to migrate to injury sites in the body.	Poor engraftment efficiency, suboptimal cell retention at the wound site, potential risk of malignancy and immunogenicity, huge costs of manufacturing, resourcing, and preservation of cell lines.
Growth factor therapy	Pivotal role in modulating and coordinating cellular and molecular events during all phases of wound healing.	Continuous administration or high doses to exert the desired effects due to the low GF *in vivo* stability.
Gene therapy	Stable nature with long shelf life of cDNA, production of protein of interest *in situ*, ease of large-scale production and low-cost.	Lack of clinically approved chemical vectors and potentially immunogenic viruses.
Topical antimicrobials	Relatively easy to use, widely available, have less risk of resistance, higher concentration at the target site and fewer systemic adverse effects than systemic antimicrobials.	Cytotoxic effects (antibiotics < antiseptics) that are influenced by the type of cell culture, number of cells, time of exposure, concentration of the antimicrobial.
Negative pressure wound therapy	Reduced frequency of dressing changes, reduced pain at dressing's changes, reduces wound odor, control of exudate, reduced infection risk. Provides a moist environment and exudate removal which help establish fluid balance, a potential decrease in wound bacterial load and an increase in the blood flow and tensile strength.	Can cause damage or rupture vessels due to the force of negative pressure, may cause trauma and bleeding.
Hyperbaric oxygen therapy	Restores oxygen into the wound, increases fibroblast proliferation and collagen deposition, improves re-epithelialization, angiogenesis, and capability of bacterial killing.	Includes barotraumatic lesions (middle ear, nasal sinuses, inner ear, lung, teeth), oxygen toxicity (central nervous system, lung), confinement anxiety, and ocular effects (myopia, cataract growth).

Table 18: General benefits and drawbacks of different drug delivery systems in wound healing.

	Benefits	Drawbacks	References
Micelles	Good colloidal stability, greater cargo capacity, biocompatibility, non-toxicity, and controlled drug release.	Rapid clearance from circulation, no convenient method of sequestering and delivering DNA and RNA, small or non-existent gene delivery potential.	(69,190)
Polymeric NPs	Biocompatibiliy and biodegradability, increasing the stability of any volatile pharmaceutical agents, less toxic, targeted drug delivery, non-immunogenicity and nontoxicity.	Toxic degradation, toxic monomers aggregation, residual material associated with them.	(191,192)
Dendrimers	Globular-shaped and easy and controlled surface functionalization.	Toxicity associated to the presence of positively charged amine groups	(193)
Other Lipid NPs	Good stability to incorporated drugs, drug release modulation, versatile chemistry, ease of preparation and scale-up, low cost.	Low space for drug encapsulation, leading to poor drug loading capacity, and chances of drug expulsion and burst during storage.	(191)
Nanoemulsions	High drug loading ability, enhanced drug solubility and bioavailability, small droplet size, large surface area, occlusive properties, relatively easy preparation and scale-up, controlled drug release, and protection from hydrolysis and enzymatic degradation.	Instability issues, expensive process, uncontrolled accumulation of active substances in reticular dermis or subcutaneous fat, large number of surfactants/co-surfactants is required for stabilizing the nano droplets.	(69,112)
Cyclodextrins	Confers solubility and stability to poorly water-soluble drugs, low toxicity and low immunogenicity.	For some molecules, low efficiency of complexation. Very high aqueous-soluble substances generally cannot be included.	(194,195)
Liposomes	Increased moisturizing, biodegradable, non-toxic, biocompatible, structural similarities to biological membranes allow better penetration into the epidermal barrier and easier incorporation of drugs.	High production cost. It may cause rapid uncontrolled release of the entrapped drugs. It is possible to get a low encapsulation efficiency, and short half-life.	(69,98,187,196)
Inorganic NPs	Good biocompatibility, ease of production, low toxicity, scalability and high bioavailability.	Limited potential in the wound healing process- their effect is heightened when combined with other agents, their application is limited due to concerns such as long-term tissue deposition and degradation.	(69,193)