Multifunctional Enzymatically-Generated Hydrogel Platforms for Chronic Wound Application

Ivaylo Stefanov Stefanov

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Department of Chemical Engineering

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This thesis is dedicated to my beloved parents,
Maria and Stefan

"Truth is ever to be found in simplicity, and not in the multiplicity and confusion of things"

(Isaac Newton)

"Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world."

(Albert Einstein)

ABSTRACT

Chronic wounds became burdensome problem of worldwide healthcare systems, along with the increased elderly population, which is the most vulnerable risk group, predisposed to their development. Chronic wounds represent a “silent epidemic” that affect a large fraction of the population and are often regarded as a comorbid condition. Statistical surveys indicated that 1-2 % of the population in developed countries will suffer from chronic wounds during their lifetime. Contemporary clinical treatment involves a combination of techniques and procedures aiming at eradication of wound chronicity and switching the biochemical entities to normal wound healing. In this regard, wound dressings have been affirmed and widely accepted as integral part of wound healing therapies. Wound care, by using dressings dates from ancient times, when for instance ancient egyptians applied and arranged bandages. Nowadays, the market is dominated by dressings, which only function besides a simple physical barrier is to balance the wound moisture by either absorbing excess exudates or providing moisture environment. However, the multifactorial nature of chronic wounds often renders this single-factor directed therapy as low or non-effective, aggravating the patient outcome. Thus, the demands for expanding the treatment options to more effective therapy brought about the development of bioactive dressings. These dressings should not only protect the wound and control the wound moisture, but also interact with various adverse wound constituents, modulating their bio-activities in favor of healing.

Materials with inherent wound healing features are highly desireable and more attention to such materials among the research communities has lead to the design of wound dressings with improved characteristics. However, amongst the myriad novel dressings synthesized, there is still lack of universal dressing with a panel of features able to address most of the devastating chronic wound constituents. The lack of such on-market dressing, lead to huge economical burden of the healthcare systems, holding significant part of their budgets. Development of universal multifunctional dressing, appropriate for management of many types of chronic wounds will boost the health systems to minimize the costs and improve the quality of patient’s life.

This thesis develops multifunctional biopolymer-based hydrogel materials as a bioactive platform with appropriate exploitation characteristics for treatment of chronic wound. To this end, hydrogels were developed by using environmentally benign approach, based on enzymatic reactions. Intrinsically bioactive biopolymer chitosan which served as a matrix, was modified with thiol groups and further in situ enzymatically crosslinked with two different natural polyphenols. The incorporated in the biopolymer matrix polyphenols, exhibited dual role on the hydrogel performance by providing: 1) structural integrity by crosslinking the biopolymer chains; 2) bioactive features, through interaction with major chronic wound factors. The multifunctionality of the obtained materials in the treatment of chronic wounds was evaluated by *in-vitro* and *ex-vivo* experiments with chronic wound exudates. The hydrogels exhibited beneficial for wound healing properties, such as inhibitory activity against deleterious wound enzymes and antioxidant activity, and antibacterial activity coupled with biocompatibility to human skin cells.

Key words: Chronic wounds, myeloperoxidase, matrix metalloproteinases, thiolated chitosan, natural phenolics, laccase, enzymatic crosslinking, hydrogels, wound dressing

RESUMEN

Las heridas crónicas representan un perjuicio económico significativo para los servicios sanitarios del mundo entero, el cual se ve magnificado con el incremento de la población de la tercera edad, que es el grupo que presenta mayor riesgo de desarrollarlas. Las heridas crónicas son una "epidemia silenciosa" que afecta a una parte muy importante de la población y que suele estar asociada a otras patologías. Las estadísticas reflejan que el 1-2% de la población de los países desarrollados sufrirá este tipo de heridas durante su vida.

Los tratamientos actuales están basados en una combinación de estrategias dirigidas a erradicar la cronicidad de la herida y activar los resortes bioquímicos para su sanación mediante el proceso habitual. A este respecto, los apósitos para heridas están ampliamente reconocidos como una parte integral de las terapias de curación de heridas. El cuidado de las heridas viene desde tiempos remotos con el uso de vendajes por parte de los antiguos egipcios. Actualmente el mercado está dominado por apósitos que actúan como barrera física que a su vez controla la humedad de la herida ya sea por absorción del exceso de exudado o por proveer la humedad necesaria. Sin embargo, la naturaleza multifactorial de las heridas crónicas hace que esta estrategia resulte en diversas ocasiones poco o nada efectiva, agravando la situación del paciente. Por lo tanto, es necesario mejorar el tratamiento desarrollando apósitos que sean bioactivos. Estos nuevos apósitos deben, además de proteger la herida y controlar su humedad, interactuar con diversos factores negativos presentes en las heridas modulando su bioactividad en favor de una curación más rápida y eficaz.

Materiales con inherentes propiedades curativas son realmente necesarios y este creciente interés ha llevado a la comunidad científica a diseñar apósitos para heridas con mejores propiedades. Sin embargo, a pesar de la multitud de nuevos apósitos desarrollados, no se ha presentado un apósito universal con múltiples propiedades capaz de hacer frente a la mayoría de los factores que provocan la cronicidad de estas úlceras. La falta de este tipo de aposito en el mercado conlleva un gasto economico muy importante para los servicios de salud y representa una parte significativa de sus presupuestos.-El desarrollo de un apósito multifuncional y universal aplicable

a varios tipos de heridas crónicas implicaría una reducción drástica de los costes de los servicios sanitarios y mejorar la calidad de vida del paciente.

En esta tesis se desarrollan materiales multifuncionales basados en hidrogeles biopoliméricos con el objetivo de generar plataformas bioactivas con propiedades óptimas para el tratamiento de heridas crónicas. La formación de estos hidrogeles se basa en una reacción enzimática respetuosa con el Medio Ambiente. El quitosano es un biopolímero intrínsecamente bioactivo que una vez funcionalizado con grupos tiol y reticulado con diferentes polifenoles constituye la estructura del hidrogel. Estos polifenoles confieren dos propiedades al hidrogel: 1) integridad estructural por la reticulación de las cadenas de quitosano; 2) bioactividad, a través de su interacción con la mayoría de factores y patógenos presentes en las heridas crónicas. La multifuncionalidad de los materiales obtenidos para el tratamiento de heridas crónicas ha sido evaluada tanto *in vitro* como *ex vivo* con exudados de heridas crónicas. Los hidrogeles desarrollados en esta tesis muestran múltiples propiedades añadidas a las de los apósitos que actualmente hay en el mercado, y entre ellas destacan una elevada actividad antibacteriana, inhibición de enzimas perjudiciales y efecto antioxidante a la vez que presentan gran biocompatibilidad con células de piel humana.

Palabras clave: Heridas crónicas, mieloperoxidasa, metaloproteinasas de matriz, quitosán tiolado, fenoles naturales, lacasa, entrecruzamiento enzimático, hidrogel, apósitos para heridas

List of publications and author's contributions

Research articles:

I. **"Multifunctional enzymatically generated hydrogels for chronic wound application"**

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I. **"Versatile chemistry of natural phenolics to produce green engineered materials"** (oral)

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VII. **"Enzymatic synthesis of hydrogels based on thiolated chitosan and chicoric acid for chronic wound application"** (oral)

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VIII. **"Antibacterial polyurethane foam with incorporated lignin-capped silver nanoparticles for chronic wound tretament"**

A.G. Morena, I. Stefanov, T. Tzanov

257th American Chemical Society National Meeting & Exposition, March 31-April 3, 2019, Orlando, USA

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contain also cytoplasmic domain at its carboxyl end. The three fibronectin-like repeats, characteristic for gelatinases represent their gelatin-binfing domain. Most MMPs contain preserved N-glycosylated site and all contain at least one N-glycosylated domain located on the hemopexin-like domain or one of the common for all types of MMPs domains. (reproduced from[4]).

Fig.1.6. Oxidative processes in chronic wounds. ROS, which are formed during the normal metabolic processes in inflammed tissues possess important role in elimination of pathogens. Disturbed balance between the levels of ROS and their detoxifying enzymes, such as superoxide dismutases, GSH peroxidases, peoxiredoxins and catalase lead to oxidation of various biomolecules of the host organism, disturbing their function (reproduced from[5]).

Fig.1.7. Example of common type of wound dressings, used for treatment of chronic ulcers. Each dressing is appropriate for different type of wounds or different stage of the wound chronicity, depending on the wound exudate produced from the wound.

Table 1.1. Commercial dressings, offered on market from the big wound dressing manufacturers. Along with the type of dressing (alginate, foam, hydrogel or hydrocolloid) are given the correpsonding brand name and example of the most common use for each dressing.

Abbreviations list

A

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ATP	adenosine triphosphate

B

bFGF	basic fibroblast growth factor

C

CAMs	cell adhesion molecules
CD44	cluster determinant 44
CFU	colony forming units
ChA	chicoric acid

D

DD	degree of deacetylation
DFU(s)	diabetic foot ulcer(s)
dG	deoxyguanosine
DM	diabetes mellitus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picrylhydrazyl

E

ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EPS	extracellular polymeric substances

ESWT	extracorporeal shockwave therapy

F

FTIR	Fourier transformed infrared

G

GA	gallic acid
GAGs	glycoasminoglycans
GSH	glutathione
Glu	glutamic acid
Gly	glycine

H

HA	hyaluronic acid
HARE	hyaluronan receptor for endocytosis
HIF 1α	hypoxia inducible factor 1α
Hys	hystidine

L

LFUS	low-frequency ultrasound
LYVE-1	lymphatic vessel endothelial hyaluronan receptor

M

MMP(s)	matrix metalloproteinase(s)
MNA	mercaptonicotinic acid
MPO	myeloperoxidase
MT-MMPs	membrane-type MMPs

N

NADPH	nicotineamide dinucleotide phosphate reduced
NB	nutrient broth

P

PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PMN	polymorphonuclear neutrophil
PrU(s)	pressure ulcer(s)
PU	polyurethane

Q

QCM	quartz crystal microbalance

R

RNAses	ribonucleases
ROS	reactive oxygen species

S

SC	stratum corneum
SEM	scannig electron microscopy
SD	standard deviation
SPARC	secreted protein acidic and rich in cysteine

T

TCS	thiolated chitosan
TGA	thioglycolic acid
TGF-β	transforming growth factor β

TIMPs	tissue inhibitors of matrix metalloproteinases
TLR3	toll-like receptor 3
TLR4	toll-like receptor 4

U

UV	ultraviolet
UVC	ultraviolet light C

V

VEGF	vascular endothelial growth factor
VLU(s)	venous leg ulcer(s)

W

WHO	world health organization

1. Introduction

1.1. Pathophysiology of chronic wounds

Chronic wounds, referred to also as chronic, hard-to-heal[6], or non-healing ulcers[7] are those which are not healing in a normal fashion and time frame. Usually, wounds which are not healing within three months are termed chronic. Due to the demographically aging population worldwide, chronic wounds became globally increasing health concern with huge economical burden and dedicated medical care. In Europe and the U.S.A. alone the annual cost for treatment of chronic wounds is as much as 10 billion dollars.

Statistics points out that 5-year mortality rates of patients with different chronic ulcers is similar or even worse than many common types of cancer. For instance, significantly higher 5-year mortality rates were observed in patients with diabetic or ischemic ulcers compared to these with prostate or breast cancer. In addition, world health organization (WHO) estimated that 5 year-mortality rate for patients with diabetic foot-related amputations is about 50 %[8]. Recently, many contributing factors and pathophysiological mechanisms of wound chronicity has been clarified, which in turn encouraged researchers to seek for novel strategies to treat hard-to-heal wounds. In this introductory section are discussed the structure and functions of the skin - the main substrate for occurrence and healing of wounds, the physiology of the normal (acute) wound healing and the pathophysiology of chronic wounds with their most significant contributing factors.

1.1.1. Skin and its function

Skin, the main substrate for occurrence and healing of injuries, is the largest organ of the human organism with an average total area of 2 m^2 in adult person. Skin is accounting approximately 15 % of body weight and is receiveing one third of the circulating blood volume. The skin with its accesory structures (nails, hair and gland, etc.) is referred to as the integumentary system[1]. The skin covers the human body and serves as a boundary between the human organism and the environment, thus isolating the body from the external environment and concomitantly allowing

dynamic interactions. Due to the topological position of the skin and the resultant interaction with the environment, several functions with big importance can be pointed out:

- *Protection*. The skin protects against uvtraviolet (UV) light and abrasion. It also serves as a barrier for microbial invasion and prevents dehydration by reducing water loss from the organism.
- *Sensation*. The skin possess sensory receptors, which function is to detect heat, cold, pressure, touch and pain.
- *Temperature regulation*. The regulation of body temperature is achieved through the control of blood flow through the skin and the activity of sweat glands.
- *Vitamin D production*. When exposed to UV light, the skin produces a molecule that can be transformed into vitamin D.
- *Excretion*. Small amounts of waste products are lost through the skin and in gland secretions.

The primary function of the skin is to isolate the underlying tissues and visceral organs and to provide the sensation as a part of the sensory function and thus to transfer an information about changes in different parameters of the environment and the interactions of the human organism with surrounding objects. The skin barrier protects the human organism from external threats such as infectious agents, allergens, chemicals and systemic toxicity. From the other hand, the skin helps for maintaining the homeostasis and protection of excess water loss from the body.

At the cellular level, the skin comprises three different layers: epidermis, dermis and hypodermis. The keratinocytes, which are the primary cells of the epidermis form five distinct epidermal layers, termed strata. Up from bottom, the layers are called *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum* and *stratum corneum* (SC)[9]. The skin barrier function depends mostly on the structure of the uppermost layer, the *stratum corneum.* SC consists of lipid–depleted corneocytes, dispersed in a lipid-enriched ECM. The main lipid classes in SC are ceramides, cholesterol and free fatty acids. These lipids form highly organized 3D stacked densely packed lipid layer, which play crucial role in skin barrier function. Impairment of the skin barrier

function can lead to different pathological conditions, arising from the skin itself to systemic toxicity of the organism. The dermis is composed mainly of fibroblasts, which actively migrate during proliferative phase to form granulation tissue. Two distinst subpopulations of fibroblasts have been found in dermal tissue - reticular and papillary fibroblasts. These cells occupy distinct niches in the dermis and have differences in secretion of several ECM components, such as collagen, decorin and fibromodullin. These subpopulations differ also in their response to growth factors, and the levels of cytokines, proteases, growth factors, MMPs and TIMPs secretion[10].

The extracellular matrix

It is well known that extracellular matrix (ECM) plays important role in wound healing process as it serves as a mediator between the cells and matrix proteins leading to dynamic reciprocity[11,12]. The ECM is three dimensional non-cellular structure, ubiquitous in all types of mammalian tissues and is essential for the cell life. Specialized cells are surrounded by the ECM to form all tissue types at tissue level of organization, including the epithelial and connective tissues, which form the skin. The biochemical composition and physical features of the ECM are well known to participate in regulation of various cell functions[13]. Different tissues are composed of unique and highly specilaized ECM species and organization, which allows ECM to carry out tissue-specific roles. The biological activities of the ECM are mediated by cell-surface receptors called integrins. Integrins provide a mechanical link between matrix components and the cytoskeleton and transduce a remarkable variety of signals either alone or in collaboration with growth factor receptors[14,15].

The ECM consists of numerous matrix macromolecules, which proportion and precise structure vary from tissue to tissue[16]. The ECM constituents can be divided into fiber and nonfiber-forming structural molecules and “matricellular proteins”, the latter being responsible for modifying the cell-matrix interaction. The nonfiber structural units include glycosaminoglycans (GAGs), such as hyaluronic acid (HA), dermatan sulfate and chondroitin sulfate and proteins heavily glycosylated with GAGs, which are called proteoglycans, such as decorin, versican, lumican and dermatopontin. Their function is creating a charged, dynamic and osmotically active space. The

fiber-forming structural molecules define the stiffness and elasticity of the skin. In human skin, the major fiber-forming component of the ECM is collagen, which now is well known as a superfamily of closely related, yet genetically heterogenous proteins. Other fiber-forming ECM constituents include: elastin, fibrillin, laminin, proteoglycans, heparan sulfate amongst others[17]. The matricellular proteins are a group of disparate secreted proteins, which normally are not expressed in adult healthy tissue, however their activity is elevated in injuries. Matricellular proteins does not perform any structural role, but rather regulate in autocrine or paracrine-signaling way cell-ECM interactions. Matricellular proteins include thrombospondin, osteopontin, secreted protein acidic and rich in cysteine (SPARC), tenascin-C and fibulin[18].

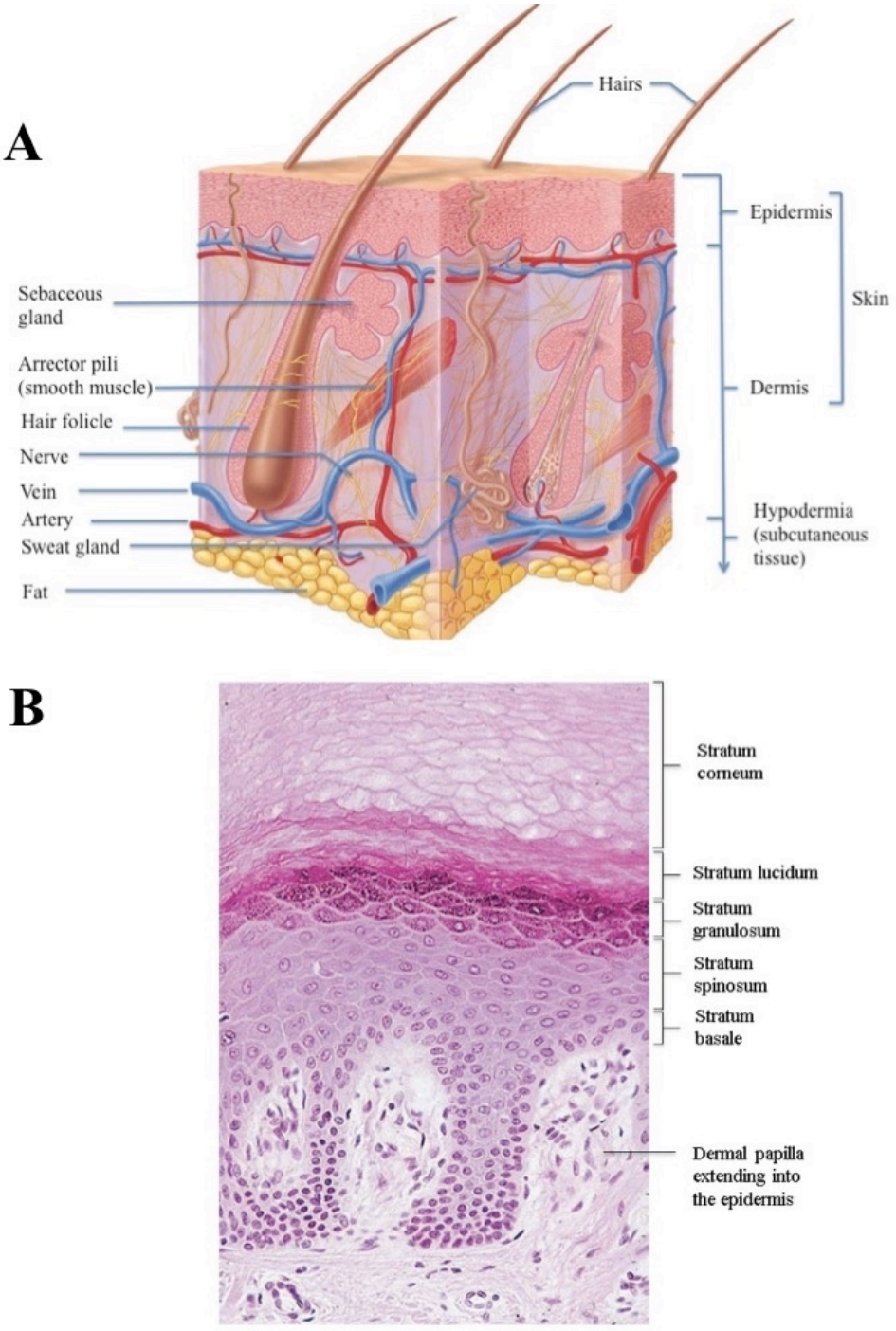

Figure 1.1. Anatomy of the skin: A) Organization of the integumentary system; B) Hystology of the epidermis. (reproduced from[1])

1.1.2. Acute wound healing

When the skin barrier is disrupted, as a result to any external impact, e.g. burn, trauma, or pressure damage the function of skin is no longer adequately performed[19]. After wound appears on the

skin, the organism aims to regenerate its integrity in order to restore its function. Wound healing is well-orchestrated and time resolved dynamic process, which can be spatio-temporarily separated into four distinct, although still overlapping phases. The first phase, not considered as a distinct phase by some authors is called hemostasis, followed by the inflammatory and proliferation phases to accomplish the healing process with the terminal remodelling phase. Each of the wound healing phases has its own physiological resolution and the difference stems mainly from the active cells, included in each phase and from the produced by these cells specific biomolecules (Figure 1.2). Wound healing is immensely complex process and it represents the evolutionary layering of numerous genetic, epigenetic and molecular processes for accomplishing this goal. Detailed understanding of wound healing physiology is crucial for the effective clinical management of the suffering patient.

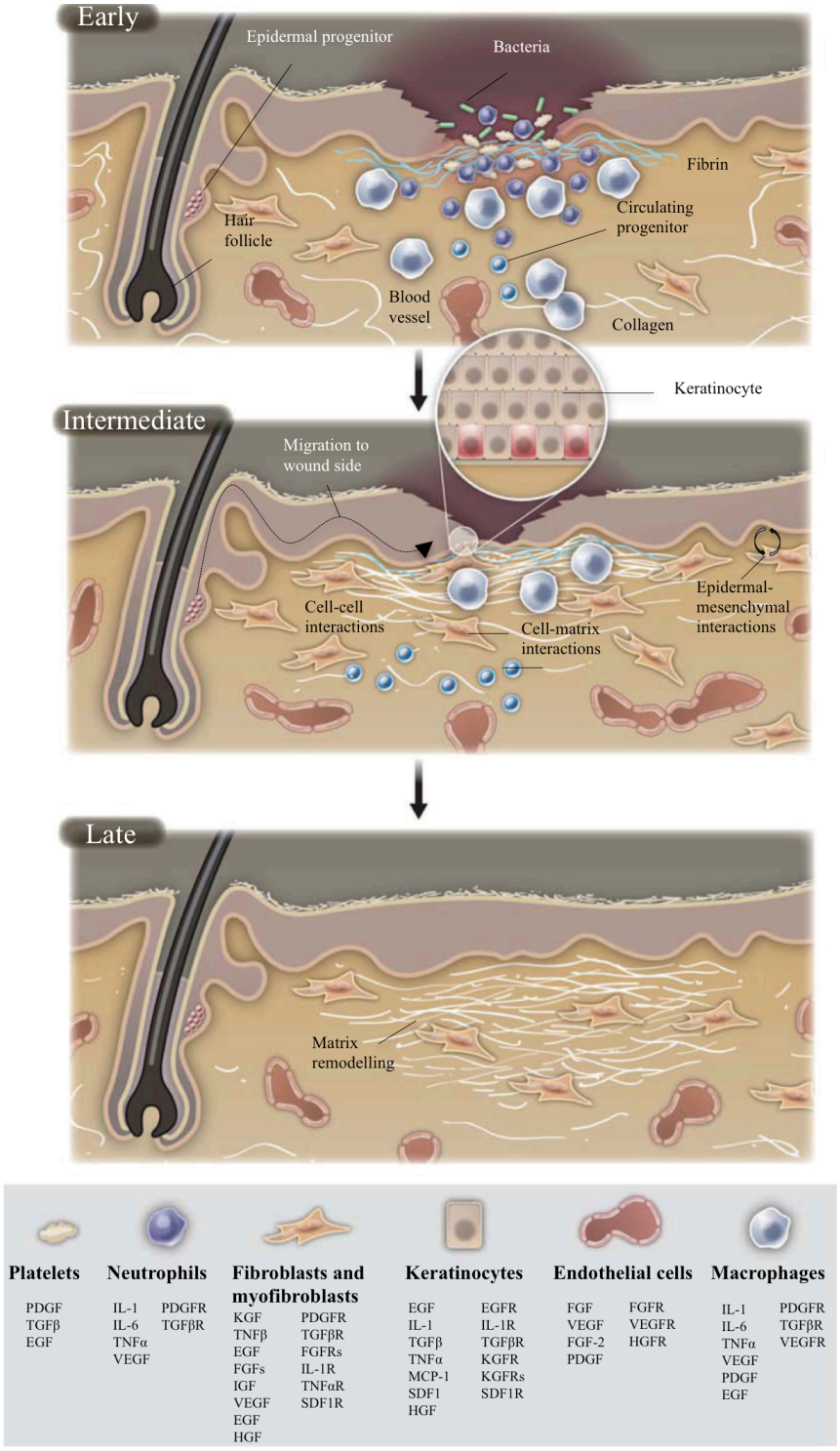

Figure 1.2. Molecular and cellular mechanisms in normal skin wound healing. Cellular and molecular mechanisms of wound healing are depicted in three conditional stages. In the early stages hemostasis and activation of keratinocytes and inflammatory cells are predominant events. The intermediate stage is characterized by proliferation and migration of keratinocytes, proliferation of fibroblasts, matrix deposition and angiogenesis. During the late-stage, scar formation and restoration of skin barrier through remodelling of ECM takes part. In this stage the closure of the

wound is driven by the recruitment of multiple cell types and secretion of numerous growth factors, cytokines and chemokines (reproduced from [20]).

Hemostasis

During this initial phase, occuring immediately after cutaneous injury, the platelets are arriving into the wound side to cause blood clot formation by cascade of enzymatically catalyzed reactions in order to prevent further bleeding. Platelets within the clot release platelet-specific proteins, such as platelet factor 4, thrombin and fibrins, growth factors such as platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), angiogenic factors, adhesion molecules and cytokines/chemokines such as neutrophil-activating peptide 2, von Willebrand factor, sphingosine-1-phosphate. The main function of these molecules is to stimulate fibrin matrix deposition forming stable clot. This clot serves as a provisional matrix for migration and assembling of inflammatory cells, fibroblasts, endothelial cells, smooth muscle cells and bone marrow derived stem cells. The aggregation of platelets also induces vasoconstriction that reduces blood flow to the wound bed.

Inflammation

The inflammatory phase, also called defensive is the second stage of the healing process, which is following the initial hemostasis and lasts for about three days. It is characterized with influx of immune cells cocktail, such as macrophages, neutrophils and lymphocytes and the produced from them cytokines and growth factors. During this phase, the vascular permeability increases to allow the localization of neutrophils and monocytes to the wound site. Neutrophils function is to remove pathogens, foreign material, damaged matrix components and dead cells by a process known as phagocytosis. Neutrophils use a bunch of chemical signals to arrive to the site of injury in another process called chemotaxis and attach to endothelial cells to the nearby vessels surrounding the wound. Thereafter, by virtue of the attached neutrophils, the endothelial cells are stimulated to express specialized cell adhesion molecules (CAMs). CAMs play role of molecular linkers for neutrophil binding to the endothelial cell surface and squeeze through the cell junctions that have

been made leaky by a mast cell mediator. The culmination of the inflammatory phase is the transformation of monocytes to macrophages. Macrophages has dual impact on the proper wound healing. From one hand the pathogenic bacteria, the devitalized tissue and other debris are digested through phagocytosis which results in cleansing the wound bed from any potentially deleterious species. From the other, macrophages secrete cytokines (such as IL-1 and IL-6) and growth factors (such as PDGF, TGF-ß, tumor necrosis factor) which is essential for the proper evolution of the next wound healing stages[21,22].

Proliferation

The proliferation phase of the healing process is characterized with migration of fibroblasts and keratinocytes from the wound edges to the wound bed proximity and subsequent proliferation to build new tissue and restore the skin integrity. The first step in this stage is the migraiton of keratinocytes over the disrupted dermis. During the next step, in a process known as angiogenesis, new blood vessels form and the fibrin matrix is replaced with granulation tissue to generate a new substrate for keratinocyte migration. The key cells in this stage are the fibroblasts which form the ECM by producing fibronectin and proteoglycan. The fibroblasts are highly responsive to chemical mediators released by the immune cells during the inflammatory phase.

Remodelling

The final and the longest stage of the wound healing process, which is called remodelling, normally begins 2-3 weeks after the injury and extends for one year or more. It is characterized by wound contraction and deposition of collagen. During this stage, the processes accompanying the previous stages tend to slow down and terminate. Most of the cells, such as macrophages, endothelial cells and myofibroblasts recruited in the previous stages undergo apoptosis or leave the wound[23].

Depending on the way of wound closure, wound healing is classified as primary, secondary, and tertiary intention. Primary intention of healing takes part, when the wound edges are approximated by sutures, staples or glue. The wounds are characterized with clean wound bed

and tissue repair usually progress without complication and tends to heal rapidly. Example for primary intention of wound healing are surgical insicions. Secondary intention of healing is characterized with extensive tissue loss and poor approximation of the wound edges. As a result of the pathology in the proliferation stage the wounds fill with an extensive granulation tissue. The defects such as infected wounds and burns can heal in this manner. Healing by tertiary intention includes a combination of primary and secondary intention and is usually performed by the wound care specialist in order to minimize the risk of infection. In the tertiary intention the wound initially undergoes debridement and observed for a few days to confirm that no infection has been developed before the wound is surgically closed[24].

Wounds can be classified also based on the depth of the injury, depending on the skin layers affected. According to this classification wounds are subdivided into: 1) superficial – when there is loss only of epidermal tissue; 2) partial thickness – epidermis and dermis are involved and 3) full thickness – underlying subcutaneous fat and possibly deeper tissues, such as bone tissue in addition to the epidermal and dermal layers are affected[25].

Different factors, ranging from the human organism itself to environmental factors can have impact on wound repair. The factors, which influence wound healing can be generally classified on local and systemic factors. Among the local factors oxygenation is important in cellular metabolism and especially in cell energetics by means of ATP production and is critical in all wound healing stages. Oxygen prevents wounds from infection, induces angiogenesis, stimulates migration and proliferation of keratinocytes and fibroblasts and collagen synthesis and promotes wound contraction. Other local factor with big impact is infection. Depending on the concentration of bacteria and the overall host response, the magnitude of wound infection can be classified as contamination, colonization, local infection/critical colonization and/or spreading invasive infection. Contamination is when there is presence of microorganisms without their replication, while colonization involves their replication without tissue damage. Local infection or critical colonization corresponds to the state when signs of local tissue response occur as the immune system recognize jeopardizing levels of replicating microorganisms[26].

1.1.3. Chronic wounds with impaired healing

Wounds, which are stalled in the inflammatory phase for prolonged period (>4 weeks) are usually classified as chronic wounds. Prolonged inflammation seizes further wound resolution towards proliferation and remodelling (Figure 1.3.). Chronic wound biochemistry in all its forms significantly deviates from the normal wound repair process. Many factors can be prerequisites for wound healing failure, such as environmental factor, age, comorbidities and underlying pathologies. In most cases, chronic wounds appear as a consequence of underlying pathology, such as diabetes mellitus, venous insufficiency and different neurological disorders. In the common case, chronic wounds feature prolonged inflammatory phase and similar molecular basics.

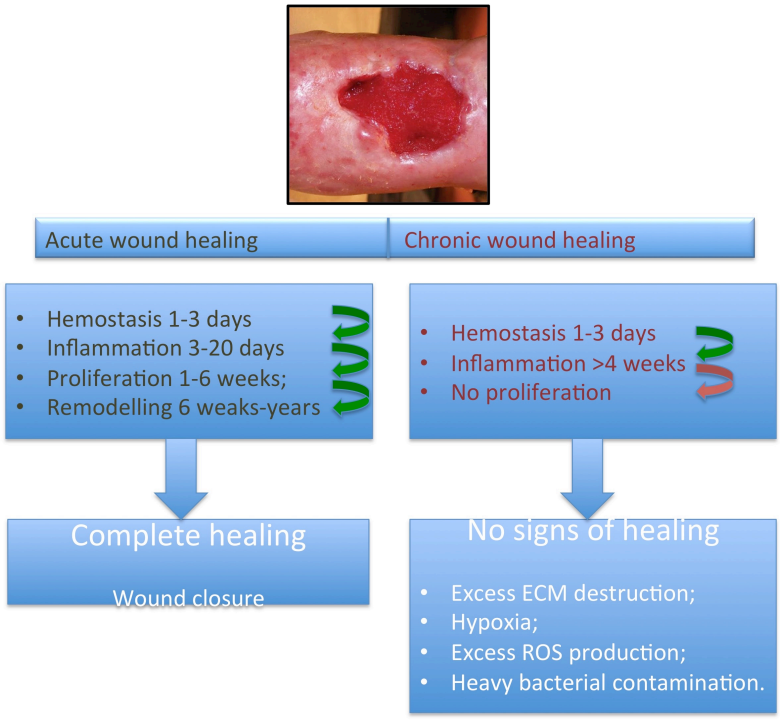

Figure 1.3. Acute vs. chronic wounds. The illustration depicts the differences between acute and chronic wound healing. Acute wounds are characterized by four overlapping phases, following predictive time-frame and ultimate in complete wound closure, while chronic wounds are stalled in the inflammatory phase with no progress towards healing.

Despite of this common biochemical characteristics, chronic wounds are usually classified on etiological principle, depending on their primary cause of the underlying disease. In the literature, three major types of chronic wounds were described, namely diabetic foot ulcer, pressure ulcer

and venous leg ulcer, representing more than 99 % of all types of chronic ulcers in humans. Other atypical and less frequently found chronic wounds include pyoderma gangrenosum, vasculitis and squamous cell carcinoma. Each type of chronic ulcer, presents specific pathology which should be considered in choosing the optimal treatment strategy.

Diabetic foot ulcers

In 2014 the worldwide diabetes mellitus (DM) cases were estimated to reach 387 million people and this number is expected to reach 592 million people by 2035 if not efficacious treatment is developed. In the U.S.A. 26 million (8 % of the population) people are diagnozed with DM and 2 million new cases are registered each year. DM refers to a group of metabolic disorders with hyperglycemia (high blood glucose) caused by imperfection in insulin release, insulin action or combination of both. Complications, related with diabetic foot ulceration are one of the most significant and devastating in patients with DM, affecting around 25 % of patients during their lifetime and associated with lower limb amputations and high rates of mortality. Around 80 % of the cases with lower limb amputation associated with diabetes are as a consequence of foot ulceration complications[27]. Indeed, more than 15 % of the diabetic foot ulcers (DFUs) result in amputation. According to WHO statistics, around 250000 DFU related amputations are performed annually in Europe, while in the U.S.A. 71000 patients with DFUs were subjected to lower limb amputations during 2010. Neuropathic complications as a consequence of diabetes was found to be the most significant predictor of foot ulceration due to the loss of protective sensation, nevertheless DFU can occur as a result of peripheral arterial disease or trauma. In addition, patients with DFUs are highly susceptible to bacterial invasion. The infection can spread on their foot and ruin their life in a remarkably short time: sometimes just within a few hours[28]. In the European countries the cost for the treatment of DFU varies between EUR 5000 and 8000 and the annual cost for unhealed ulcer was estimated to reach EUR 20000. In the cases where limb amputation is required, the cost can reach EUR 10000-32000. The total cost per DFU patient in Spanish hospital units was estimated to reach EUR 7633[29].

Pressure ulcers

Recent studies revealed that the prevalence of pressure ulcers (PrUs) among hospitalized patients in Spain vary between 8 and 16 %[30]. PrUs, known also as decubitus ulcers or bedsores are caused by impaired blood supply and tissue malnutrition as a result of prolonged and unrelieved pressure and/or shear to an area of the body, most commonly over body prominences. In majority of cases, PrU develops on the lower half of the body – around the pelvis or on the lower limbs with heel ulceration becoming more common[31]. Patients, at high risk for development of PrUs are older adults and chidren, obese and underweight patients, as well with different neurological conditions, such as multiple sclerosis, spinal cord injury, motor neurone disease, stroke amongst others[27]. The average costs per month for such cases was reported to be $4745 to the Canadian health system. The total annual cost for management of a single PrU can be as high as $70000 in the U.S.A., while the annual expenditures of the American healthcare system for the treatment of PrU is estimated to reach $11 billion annually. In a study conducted during the year 2007, it was estimated that the annual cost for PrUs treatment in Spain was EUR 461 million, which is roughly 5 % of the total healthcare expenditure[30].

Venous leg ulcers

Venous leg ulcers (VLUs) are accounting for around 60-80 % of all foot ulcerations. The prevalence of VLUs is between 0.18 and 1 %, and among the elderly population aged over 65, the prevalence rises up to 4 %. Venous leg ulceration appears as a result of sustained venous hypertension which stems from chronic venous insufficiency due to dilated veins or damaged valves in leg veins. The altered permeability of the blood vessel wall lead to leakage of fibrin and various plasma components into the perivascular space. Accumulation of fibrin delays wound healing by down-regulating collagen synthesis, formation of fibrin cuffs in the pericapillary space which hamper the normal blood vessel function, and entrapment of blood-derived growth factors[32]. In most cases, the venous leg ulcer arise over the medial aspect of the lower limb between the lower calf and the medial malleolus. The patients suffer from edema, venous dermatitis, pigment deposition (combination of hemosiderin and melanin) and

lipodermatosclerosis[33]. The cost for treatment of VLU in the U.S.A. was estimated to reach $10563 for the cases where no recurrence was observed during the follow-up, while for the unhealed ulcers the total costs reached a mean value of $33907[34].

1.1.4. Factors contributing for wound chronicity

Despite their different etiology, one common underlying mechanism for the failure of wound healing progression exist for all types of chronic wounds, that is the prolonged inflammation. However, the complexity of wound physiology and pathophysiology, the dynamic interactions at molecular, cellular and tissue levels with fluctuations in wound microenvironment and lack of adequate animal models, resembling chronic wounds in humans hindered further development of unified theory for the treatment of all types of chronic wounds. Nevertheless, in many studies it was demonstrated that most of chronic wounds feature elevated levels of MMPs, myeloperoxidase, reactive oxygen species and in addition heavy bacterial colonization as a result of the prolonged exposure of the wound to the environment. Chronic wounds suffer also from senescense of important cells, participating in the proper wound healing process, such as fibroblasts, keratinocytes, endothelial cells sand macrophages[35].

1.1.4.1. Myeloperoxidase

Myeloperoxidase (MPO, EC 1.11.1.7) is an oxidative enzyme, which due to its green colour was originally named verdoperoxidase. MPO is encoded by a single gene (approximately 14kb in size), composed of 11 introns and 12 exons, and located on the long arm of chromosome 17 in segment q12–24[36]. MPO with 146 kDa molecular weight is highly cationic (isoelectric point>10), arginine rich iron-containing glycosylated dimer heme protein[37], consisting of two 73 kDa monomers linked via a cysteine bridge at Cys153. It is stored in the azurophilic granules of the polymorphonuclear neutrophiles (PMN), which are the major cells recruited on the site of local infection as a response of the immune system to inflammation. The optimum pH of the enzyme is 5.5, although it is active yet over a wide range of pH in presence of high concentrations of

hydrogen peroxide and tyrosine[38]. Apart from neutrophils, the presence of MPO have been proved in monocytes, macrophages and lymphocytes.

MPO can be involved in two distinct catalytic cycles, namely chlorination and oxidation cycles (figure 1.4.). During the chlorination catalytic cycle, MPO catalyzes the turnover of chlorine anions to hypochlorous acid (HClO) through the active redox intermediate compound I. Using the same pattern, MPO can oxidize other anions like bromine and iodine and the pseudohalyte isothiocyanate to the corresponding hypohalous acid. MPO also performs a peroxidase cycle in which Compounds I and II oxidize the substrate to its radical in single-electron steps. Compound I generally reacts faster, and the Compound II reaction limits the rate at which the enzyme turns over[39]. HClO, generated during the chlorination cycle is the most potent antibacterial compound, produced by the human organism and effectively participates in elimination of invading pathogenic bacteria[40].

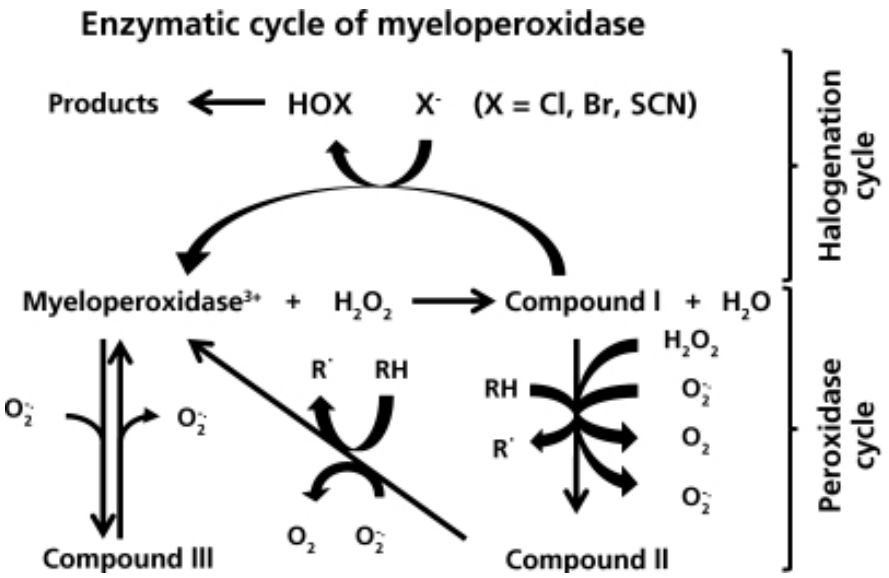

Figure 1.4. Shematic representation of the MPO enzymatic cycles. MPO catalytic activity is presented by two distnict catalytic cycles-halogenation and peroxidation cycles. Initial oxidation of the iron (III) by H_2O_2 gives rise to the intermediate Compound I, which represents iron (IV) species. During the halogenation cycle, hypochalous or hypothiocyanous acids are generated from the corresponding halogenous or hypothiocyanous ions through Compound I with subsequent conversion to the ferric form of the enzyme. During the peroxidase cycle Compound I undergoes two successive one-electron reductions to Compound II, which eventually retains the oxy-ferryl center. The iron (III)

form of the enzyme can be involved in superoxide radical-mediated reduction to give Compound III. (reproduced from[3])

Despite its natural power to kill bacteria by causing oxidation of molecular constituents, i.e. lipids, proteins and nucleic acids, elevated levels of MPO has been associated with the pathophysiology of many diseases. The detrimental for the human organism consequence of MPO action is related to its ability to oxidize not only the essential bacterial polymers but also the biopolymers of the host organism. Higher concentrations of MPO can serve as a marker of inflammation, related with severe diseases. Elevated levels of MPO has been detected in different neurodegenerative disorders such as multiple sclerosis[41], atherosclerosis[42,43], ischemic brain injury[44], Alzheimer disease[45], cardiovascular diseases such as chronic heart failure[46], myocardial infarction[47], peripheral vascular disease[48], coronary artery disease[49] and some types of cancer such as ovarian cancer[50] amongst others.

In some pathological conditions, tissue levels of MPO can be deviated from the normal concentrations. For instance, primary MPO deficiency has genetic origin, while secondary MPO deficiency occurs in various clinical situations, such as lead intoxication, renal transplantation hematological neoplasms, disseminated cancers, thrombotic diseases, diabetes mellitus, iron deficiency, pregnancy among others[51]. MPO can be also expressed in elevated concentrations in infected wound environment and as a consequence additionally deteriorate the normal wound healing process, increasing the oxidative stress. MPO can serve as a marker of infected wounds, where its elevated levels can be detected in wound fluids and thus can provide information for the overall status of the wound and for monitoring of the wound healing progression[52]. Different materials was proposed from several authors for detection of wound fluid MPO activity, such as immobilized MPO substrate on silica plate[53], electrochemical sensor[54], cyclodextrin-based luminiscent nanoparticles[55]. It was demonstrated, that accumulation of H_2O_2 in inflammed tissue in presence of MPO, released from PMNs can exacerbate the inflammatory response and promote epithelial injury[56].

1.1.4.2. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a big class of zinc-dependent and calcium-activated endopeptidases, which are ubiquitously distributed in various mammalian tissues. MMPs, which belong to the metzincin superfamily of metalloproteinases are multidomain proteins with globular catalytic domains that are approximately 130–260 residues in length[57]. Up-to-date a total of 24 different enzymes, classified as MMPs were discovered in different human tissues. Despite of differences in their function and substrate preferencies, MMPs share similarities in their domain organization. The active center of all types of MMPs consist the preserved zinc binding motif HisGluxxHisxxGlyxxHis. Majority of MMPs possess signal peptide, which is followed by four distinct domains, the N-terminal prodomain (propeptide), catalytic domain, linker (hinge) region, and C-terminal hemopexin-like domain (Figure 1.5.). Membrane-type MMPs (MT-MMPs) possess additional transmembrane domain which serves as an anchor in their interaction with the cell membrane and some of the membrane type MMPs possess also cytoplasmic domain at the carboxylic terminal group.

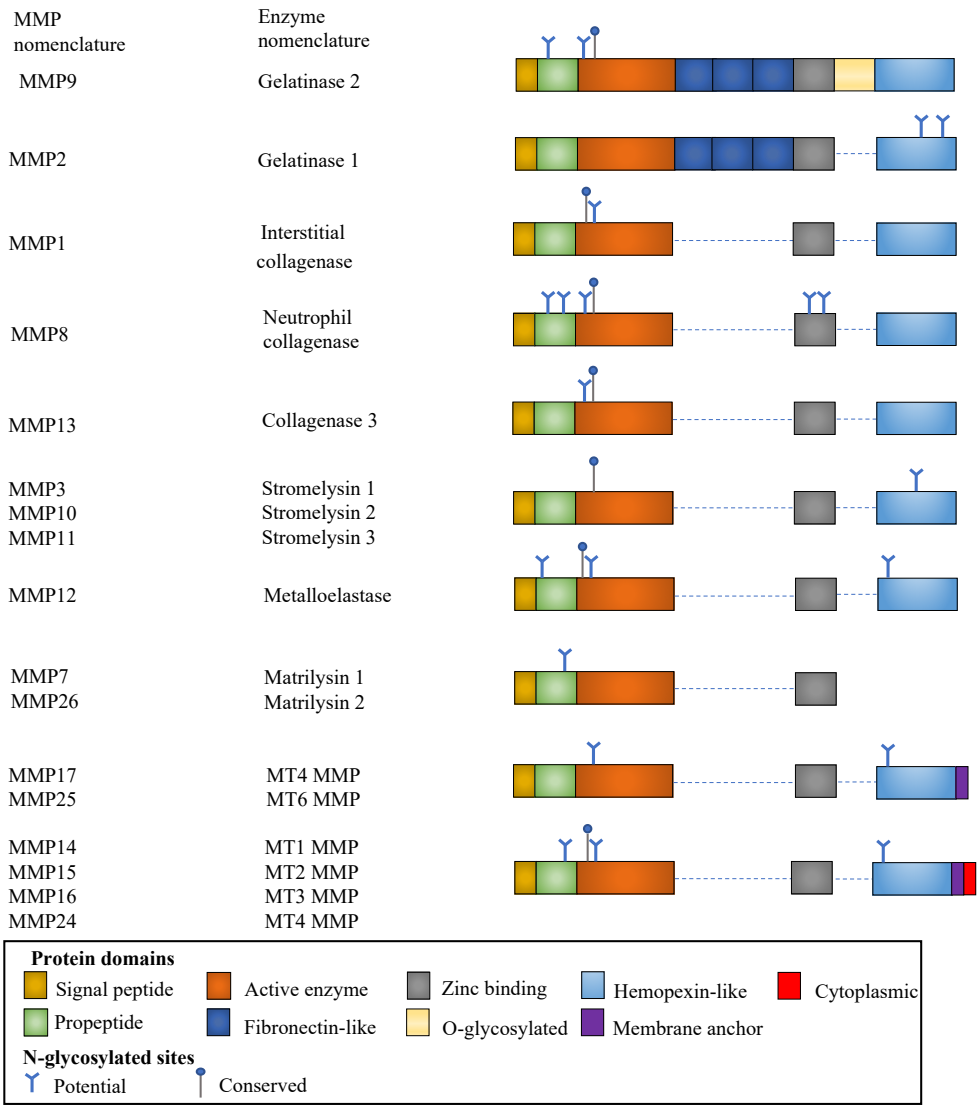

Figure 1.5. MMPs domain structure. Multidomain structure of MMPs consists of pro-domain (signal peptide and propeptide), active domain, zinc-binding domain, hemopexin-like domain (without MMP7 and MMP26). All MT-MMPs contain membrane anchor domain and some of them contain also cytoplasmic domain at its carboxyl end. The three fibronectin-like repeats, characteristic for gelatinases represent their gelatin-binding domain. Most MMPs contain preserved N-glycosylated site and all contain at least one N-glycosylated domain located on the hemopexin-like domain or one of the common for all types of MMPs domains[4].

Most MMPs has low specificity and can process wide range of substrates, such as proteases, chemokines, growth factors, cytokines, adhesion molecules and matrix proteins, which are the structural elements of the extracellular matrix (ECM)[58]. The most important ECM proteins, which serve as MMPs substrates are collagens, proteoglycans, elastin and laminins amongst others[59]. The activity of MMPs is tightly regulated at three different stages: 1) transcription; 2) zymogen

activation; and 3) inhibition of active forms by tissue inhibitors of MMPs (TIMPs)[60]. The active site of MMPs consists of one glutamine (Glu) and three histidine (His) residues, where the Glu residue assists in the nucleophilic attack at the peptide carbonyl group of the substrate by a zinc-coordinated water molecule, while the His residues are coordinated to the catalytic zinc ion[61]. MMPs are released in the tissues in their "proactive" zymogen form, and are activated through the highly conserved domain, called cysteine-switch mechanism. This mechanism is based on disruption of the bond between cysteine thiol residue and the catalytic zinc ion to derive the active form of MMPs[62]. The thiol residue of pro-MMPs cysteine-switch can be oxidized by MPO-generated HOCl which results in activation of the pro-forms into their active MMPs, envisaging the tight connection between MPO and MMPs elevated activities[63,64].

The activity of MMPs is supressed by their natural inhibitors - the beta-macroglobulins and the tissue inhibitors of MMPs (TIMPs), which action is highly essential for the functional balance of the ECM synthesis and turnover. Four types of TIMPs have been identified in vertebrates, namely TIMP-1, TIMP-2, TIMP-3 and TIMP-4 each binding MMPs in a 1:1 stochiometric ratio[65]. The disturbed ratio between MMPs and their natural inhibitors manifests in various pathophysiological conditions and related diseases, such as cancer, atherosclerosis, osteoarthritis, cardiovascular and heart disease, multiple sclerosis amongst others[66]. Similarly, wound chronicity is related with disturbed ratio TIMPs/MMPs in favor of the enzymes, which eventually results in uncontrollable excessive degradation of the ECM[67].

Expression and activation of MMPs plays crucial role in all stages of wound healing by modifying the wound matrix, allowing for cell migration and tissue remodelling[68]. Nevertheless, the overexpression of MMPs accompanying wound chronicity can be deleterious for the patient outcome, due to the excessive degradation of ECM constituents. The activity of total MMPs, measured in chronic wounds was found to be up to 30 times greater, compared to this in acute wounds[69]. Among the big MMPs family, MMP-2 (gelatinase 1) and MMP-9 (gelatinase 2) have been found as constituents of healthy skin tissue, while most of MMPs are activated in pathological conditions. In chronic wounds, high collagenolytic activity have been detected due to the elevated levels of MMP-1 (collagenase 1) and MMP-8 (collagenase 2) along with decreased

level of their indogenous inhibitor, TIMP-1. MMP-1 possess broad substrate specificity, cleaving various ECM proteins and proteoglycans, including collagen, versican, perlecan, aggrecan which influence the deposition of ECM. Neutrophil-derived MMP-8 is overexpressed and activated in chronic wounds as an immune response to infection. The concentration of MMP-8 in chronic wounds can be 50-100 fold higher than in acute wounds. It cleaves collagen 1, one of the major interstitial collagens, which is providing skin tensile strength and cellular signals. Moreover, MMP-8 has potential impact on wound pathophysiology by degradation of α2-macroglobulin, α1-antiproteinase, fibronectin, growth factors, such as TGF-β and PDGF[70]. Another type of MMPs, also called gelatinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are up-regulated in chronic wounds. MMP-2 and MMP-9 have very broad spectrum of substrates, ranging from different types of collagens to their hydrolyzed products – gelatins, degrading it to smaller fragments. Nevertheless, it was found that when MMP-9 was in concentrations below the normal levels, delayed reepithelialization occurs which elucidates the importance of this particular enzyme in the normal wound healing process.

1.1.4.3. Bacterial contamination

It has been estimated, that 1 cm^2 of the skin is covered in average with 10^6 bacteria, which is variable among individuals and different physiological conditions. In literature are considered two categories of skin flora: 1) resident - bacteria, which is normally found on skin; 2) transient – bacteria which is not normally found and is removed by daily washing procedures. The most commonly found bacterial species in routine examinations are *Peptococcus*, *Staphylococcus*, *Propionibacterium*, *Streptococcus*, *Brevibacterium*, *Corynebacterium*, *Neisseria*, *Micrococcus* and *Acinetobacter*[71]. Bacterial cells are minimally invasive through the protective skin barrier, which preserves the underlying tissue intact from pathogenic interference. Barrier for bacterial invasion in skin is partially mediated by antimicrobial peptides, defensins, cathelicidins, protegrins and ribonucleases (RNAses)[72,73]. It was shown, that the metabolic products or structural components of some of the commensal bacteria of the skin microbiome, possess profound function on skin immunity. For instance, *Staphylococcus epidermidis* produce lipoteichoic acid,

which acts selectively on keratinocytes through Toll-like receptor 3 (TLR3), inhibiting inflammatory cytokine release. Disruption of skin integrity, as a consequence of lacerations, abrasions, burns or surgical interventions inevitably leads to bacterial contamination. In acute wounds the contamination is easily eradicated, by the recruited immune cells and by the secreted from them antimicrobial molecules.

When the wound is stalled in the inflammatory phase and the process does not further progress to healing, the wound bed can be heavily contaminated with pathogenic bacteria, which can eventually rise to infectious process, limb amputation and death. Despite the multiple antibacterial factors, present in the wound bed, when the bacterial population has proliferated to extent where the bacterial virulence is enhanced, the immune system is not capable anymore to adequately react to the bacterial invasion and to protect the organism from further infectious complication.

Later, when the population progress towards the wound bed, a consortium of microorganisms occur to form biofilm. The features and behavior of microorganisms, included in biofilm has considerable differences compared to their planktonic counterparts. Biofilms are heterogenous community of bacteria or fungi attached to a tissue surface and are embedded in self-produced thick, slimy barrier of hydrated extracellular polymeric substances (EPS), consisting mainly of polysaccharides, lipids, nucleic acids and proteins. This EPS acts as an adsorbent or reactant, reducing the amount of antimicrobial agent available to interact with bacterial cells, and in addition as a physical barrier, hampering the penetration and diffusion of the agent. The bacterial cells involved in biofilms are 10-1000 times less susceptible to conventional antimicrobials, such as antibiotics[74]. In general, the presence of biofilm is a signal for detrimental patient outcome, delaying the wound healing and often leading to limb amputation and mortality[75,76]. Important point for the clinicians will be clarifying whether biofilms cause wound chronicity or the already established chronic wound microenvironment renders the wound bed highly susceptible to biofilm formation and favors its development.

Chronic wounds have a complex colonizing flora that changes over time. Amongst the most commonly isolated microorganisms are the coagulase-negative staphylococci and the gram-positive strain *Staphylococcus aureus.* Among the gram-negative species, commonly isolated are

Pseudomonas aeruginosa and *Escherichia coli.* In their study, Wong et al. found that the most prevalent bacteria found in wounds were *S.aureus* (23.3 %) and *P.aeruginosa* (14.8 %) among 210 pathogenic bacteria isolates. *S.aureus* was found significantly more often in patient with chronic wounds (48.8 %) than in those with acute wounds (9.5 %)[77]. It was found that pathogeneicity enhancement between different bacterial strains in polymicrobial biofilms exist[78].

1.1.4.4. Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules that originate mainly from the mitochondrial electron transport chain[79] or their generation can be mediated by a family of reduced nicotineamide adenine dinucleotide phosphate oxidases (NADPH oxidases)[80]. These highly reactive and unstable molecules are able to oxidize nearby molecules to gain an electron to enter the ground states. A number of different ROS exist in biological systems, such as lipid peroxides, nitric oxide (NO), singlet oxygen ($^1O_2$), ozone (O_3), and hypochlorous acid (HOCl); however, hydrogen peroxide (H_2O_2), superoxide anions ($O_2\cdot^-$) and hydroxyl radicals (·OH), are the three most important ROS playing roles in biological systems[81].

Due to their high reactivity, ROS are capable of inducing damages to biological systems, including breaks in desoxyribonucleic acid (DNA) strands, inhibition of RNA and protein synthesis, mutations as a result of base modifications, protein damage including disruption of amino acid bonds and also their cross-linking, oxidation of membrane phospholipids, lipid peroxidation, disruption of membrane ion gradients, and depletion of cellular levels of adenosine triphosphate (ATP) leading to cellular dysfunction (Figure 1.6.)[82,83]. Mitochondria, possessing the highest turnover of oxygen, involving enzymes of the respiratory chain, are the specific targets of ROS. Out of the three ROS, ·OH is the most reactive and can immediately interact with any molecule in its vicinity and can remove electron, turning that molecule into a free radical and giving rise to chain reactions thereby. The ·OH radical specifically induces hydroxylation of deoxyguanosine (dG) in DNA forming 8-OH-dG, which can be a site for mutagenesis and lead to cancer formation. The $O_2\cdot^-$ anion in comparison to ·OH is less reactive, however it is able to initiate lipid peroxidation in its protonated form or inactivate certain specific enzymes. H_2O_2 has

low reactivity and higher stability, and therefore can penetrate into the nucleus and react with important components such as nucleic acids and nuclear proteins besides other cellular components such as lipids[82,84].

Elevated levels of ROS lead to oxidative stress which is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage"[85]. The mammalian and human organisms evolved antioxidant defense mechanisms to protect vital molecules from ROS and related species-provoked damage. These cellular protectants from ROS-induced damage can be the indogenously produced antioxidant enzymes such as superoxide dismutase, catalase, thioredoxin, peroxiredoxin and glutathione peroxidase[86] or antioxidants derived from dietary sources, such as vitamin C, vitamin E and carotenoids[87].

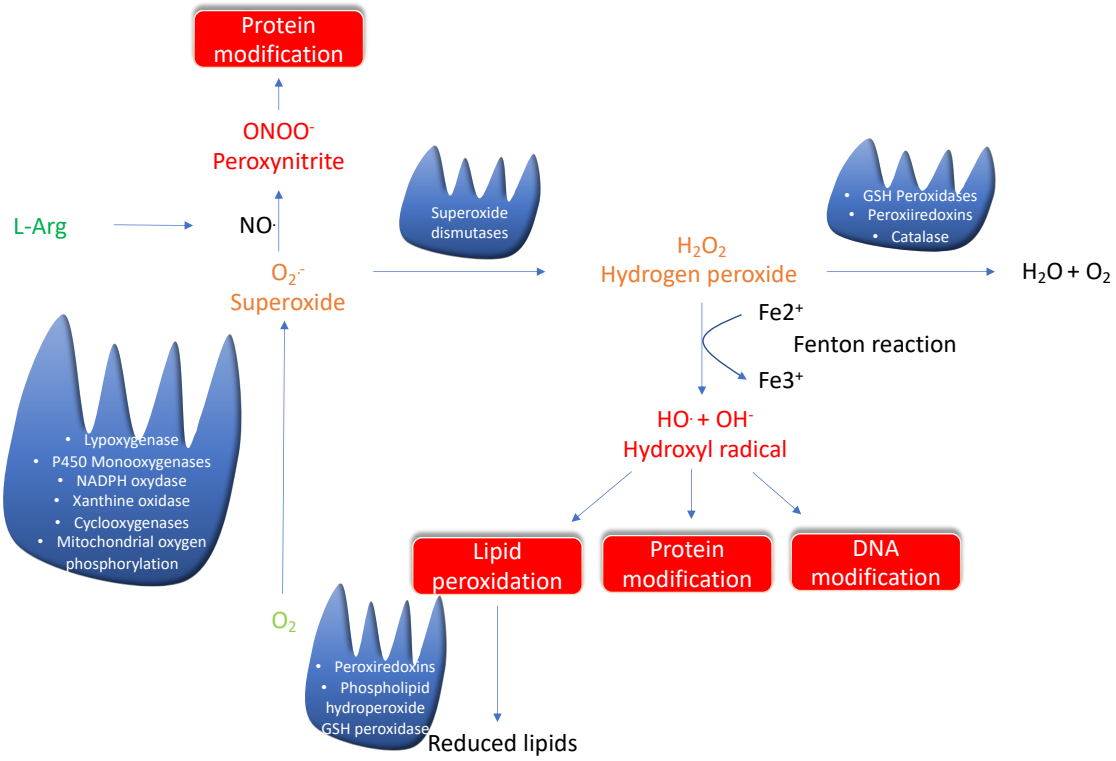

Figure.1.6. Oxidative processes in chronic wounds. ROS, which are formed during the normal metabolic processes in inflammed tissues play important role in elimination of pathogens. Disturbed balance between the levels of ROS and their detoxifying enzymes, such as superoxide dismutases, GSH peroxidases, peroxiredoxins and catalase lead to oxidation of various biomolecules of the host organism, disturbing their function (reproduced from[5]).

Beyond its apparent antibacterial role in the host organism protection[88], ROS generated mainly by mitochondria metabolism have been found to mediate many cell signalling processes and the thin line between physiology and pathophysiology seems to be the duration and intensity of the oxidants produced[89]. Signal transduction processes, mediated by ROS involve hypoxia by regulation of hypoxia-inducible factor 1α (HIF-1α), regulation of the inflammatory response by activation of the inflammasome and regulation of autophagy[90]. At physiological levels, reactive oxygen species play pivotal role in regulation of acute wound healing by facilitating hemostasis, inflammation, wound closure, and development and maturation of the ECM[5,91–93]. Although, the cells possess robust multiple system which aim in self-protection from oxidative stress, they can suffer severe damage as a result of oxidative stress in case ROS-detoxifying system is not sufficient or excessive amount of ROS are present in the wound bed. The elevated levels of ROS in chronic wounds lead to oxidation of biomolecules which disturbs their normal function and results in detrimental outcomes[94–96].

1.2. Chronic wound management

1.2.1. Organizational aspect of chronic wound management

Many health organizations are nowadays establishing programmes for comprehensive wound care, providing a full scope of services to its clientele. Choosing the most appropriate therapy is critical step in chronic wound management and requires a highly educated wound specialist with basic knowledge on wound care and relevant pathologies. The specialist must create credibility, which is accomplished by demonstrating clinical competence, organizational behaviour, critical thinking, self-confidence and ardor to collaborate and share knowledege with colleagues. These key points are crucial for successful wound therapy, minimizing the patients suffering and huge costs for the healthcare systems[97]. The contemporary holistic methodology should be consistent for all wounds regardless the wound type. It involves a consistent approach of managing all types of chronic ulcers from diagnosis to follow-up based on recognition of wound characteristics, comprehensive criteria for assessment, adequate patient and wound bed preparation, optimal treatment and proactive follow-up, aiding recurrence prevention[98]. Early and accurate wound

diagnosis is crucial for choosing the most appropriate steps for treatment of chronic wounds. Inaccurate and/or late diganosis can lead to serious complications, including permanent disability, amputations and even sepsis and other life threatening conditions. For instance, depending on the underlying pathology diabetic foot ulcers can manifest as neuropathic, ischemic or neuroischemic which differentiation is essential in the diagnosis, since each type requires different therapeutic strategies.

1.2.2. Wound bed preparation

Wound bed preparation describes a well-established concept emphasizing a systematic and holistic approach to assess and eliminate barriers to the normal wound healing process, allowing it to progress within its normal pattern. It serves as a guidance for the development of adequate treatment strategies, targeting simultaneosly the wound and the underlying disease that caused wound chronicity and facilitating the effectiveness of other therapeutic measures. To this end, therapeutic agents are optimized to accelerate endogenous healing or enhance the effectiveness of advanced therapies. The main goal of wound bed preparation is to optimize the wound microenvironment towards healing by creating well vascularized clean wound bed with littile or lack of exudates. In chronic wound, it is performed via removing senescent or abnormal cells, reducing the bacterial load and levels of exudate and enhancing the formation of granulation tissue. Local management of chronic wounds involves: ongoing debridement phase, management of exudate and resolution of bacterial imbalance.

1.2.2.1. Wound debridement

One of the most well established and efficient techniques in chronic wound care is the wound debridement. Debridement is defined as removing of devitalized, necrotic or infected tissue from the wound bed. Five variations of wound debridement are available in clinical practice nowadays: 1) biological, 2) enzymatic, 3) autolytic, 4) mechanical and 5) surgical sharp and conservative sharp debridements. The most appropriate methods for removing of devitalized tissue and cellular

debris may be detrimental for surrounding healthy tissue and therefore in many cases combination of several methods is the most appropriate.

The biological debridement consists of application of grown in a sterile environment maggots (*Lucilla sericata*) directly on the wound bed which function is to digest devitalized tissue and pathogens. Three key enzymes, participating in ECM components degradation has been identified in maggot excretions. In addition, antibacterial substances active against major wound pathogens, such as *Staphylococcus aureus*, meticillin-resistant *Staphyloccocus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* has been detected. Maggot debridement therapy is used for cleaning and disinfection of sloughy, necrotic and infected chronic wounds in patients with diabetic or pressure ulcers. Limitation of maggot wound therapy is that the larvae requires moist environment, thus dry wounds are contra-indicative[99,100].

Enzymatic debridement, most commonly achieved by the use of collagenases from mammalian or bacterial origin is a technique that is commonly used in clinical practice. Enzymatic agents are frequently used for initial debridement when anticoagulant therapy renders surgical debridement unfeasible. Collagenase is an effective, selective method of removing necrotic tissue from pressure ulcers, leg ulcers, and burns[101,102].

Autolytic debridement is a process by which the wound bed clears itself of debris by utilizing phagocytic cells and proteolytic enzymes. This process can be promoted and enhanced by maintaining a moist wound environment. It is the simplest and the most natural form of debridement, however it is slower process than the other methods. Autolytic debridement should be promoted for healing in all wounds but is generally contraindicated in infected wounds[103].

Mechanical debridement includes irrigation, wet-to-dry dressings, hydrotherapy and dextranomers. Mechanical debridement provides a rapid way to remove devitalized tissue. Major drawback of mechanical debridement is that healthy tissue can be removed along with necrotic material. This type of debridement is most commonly used for large highly exudative wounds. Nevertheless, it can be applied on small wounds by moistening necrotic eschar and facilitating their removal[104].

Surgical (sharp) debridement is usually performed for the removal of thick, adherent eschars and devitalized tissue in large ulcers. It can be also used for removing necrotic tissue rapidly when there is evidence of infection or sepsis. Sharp debridement also offers the advantadge of obtaining a tissue when infection of the deep tissues is suspected[105].

1.2.2.2. Management of exudate

All acute and chronic wounds are producing certain amounts of wound fluid, which is called exudate. It was widely accepted, that healing of acute wounds requires certain levels of exudate in order to prevent the wound bed from drying out, helping in cell migration and providing essential nutrients and growth factors to the wound. Along with this concept, occlusive dressings have been applied in order to promote the healing process by assisting the epidermal migration, alteration in pH and oxygen levels, retention of wound fluid and maintenance of electrical gradient. However as the chronic wound fluid contains elevated levels of molecular species affecting in negative way the tissue outcome, dressings which largely remove the wound exudate are more appropriate for chronic wound treatment. In case the excess wound exudate is not effectively reduced, the wound bed will become overhydrated with subsequent leakage of wound fluid in the periwound space causing further maceration and excoriation and making the skin more prone to damage. In this context, compression bandaging or dressing with high absorptive capacity are helpful in removing wound fluid, allowing growth factors to promote angiogenic response and proceed toward wound closure and concomitantly not allowing for wound fluid leakage onto healthy tissue. Dressings in different forms are available nowadays, including foams, hydrocolloids, hydrogels, transparent films and alginates.

Foam dressings are widely used for treatment of both acute and chronic wounds with various etiologies. These types of dressings are highly absorptive and thus have been applied for management of highly exuding wounds. The most commonly used foam for wound dressings is polyurethane (PU). Silicon foam is most commonly applied as an adhesive wound contact layer and less frequently – as a primary absorbent. In order to improve their perfromance, foams can be loaded with bioactives, such as silver or ibuprofen[106]. However, in a randomized controlled

trials of 12 patients with venous leg ulcers, foam dressings did not revealed superior healing rates, compared to other types of wound dressings[107].

Hydrogels are hydrophilic 3D networks, which are capable of entrapping large amounts of water and physiological fluids in its porous structure. These type of dressings cover most of the characteristics of an "ideal dressing", fullfilling desired properties, such as keeping the wound moist, while absorbing excess exudate, pain-reduction through cooling the wound surface, adhesion-free coverage of sensitive underlying tissue and potential for active intervention in the wound healing process[108]. Hydrogels are suitable for cleansing of dry, sloughy or necrotic wounds through rehydration of nonviable tissue and accelerating autolytic debridement. It was shown, that hydrogel-based dressings can absorb up to 1000 g exudate per g dressing. Disadvantage, which clinicians face when applying hydrogel-based wound dressings is the requirement for their frequent change.

Hydrocolloid dressings are made of gel-forming agents, such as carboxymethylcellulose, gelatin or pectin. Hydrocolloids form hydrophilic gel upon wound exudate contact, which facilitates autolytic debridement. This type of dressing provide occlusive bacterial and viral barrier, reducing the risk of cross-infection while at the same time using the body's own moisture to keep the wound bed hydrated for proper wound healing. Hydrocolloid dressings are appropriate for light to moderately exuding wounds such as pressure sores, minor burns and traumatic injuries. Their ability to be left on place for up to seven days, rendered hydrocolloids ideal primary dressing under compression systems[109,110].

Transparent film dressing is a thin sheet of see-through material, generally polyurethane. Due to the transparency of these dressings, the healing progress and any drainage can be monitored, while the affected area kept moist to optimize the healing process. Transparent film dressings can be used for treatment of wounds with little or lack of drainage and where dead tissue requires debridement. Due to their moisture vapor transmission capability, these types of dressings prevent the accumulation of excess moisture in the zone between the wound and the dressings, keeping the wound moisture in optimal levels[111].

Alginate dressings consist of sodium or calcium alginate, derived from seaweed. These dressings are appropriate for treatment of heavily exuding wounds, due to their capability to absorb 15-20 times more than their own weight when in contact with wound exudate. Gel forms upon Ca^{2+} ion-exchange from the alginate fiber with Na^{+} from the wound exudate. Alginates are effective for pressure or vascular ulcers, sinus tracts, wound dehiscence, surgical incisions, exposed tendons, tunnels, skin graft donor sites and infected wounds. These dressings are contraindicated for application on dry wounds, due to their poor hydration qualities. Most of alginate dressings are produced in flat sheet forms, which are generally applied on surface wounds, nevertheless alginates can be found in the form of ropes and ribbons, which are more appropriate for cavity wounds[112].

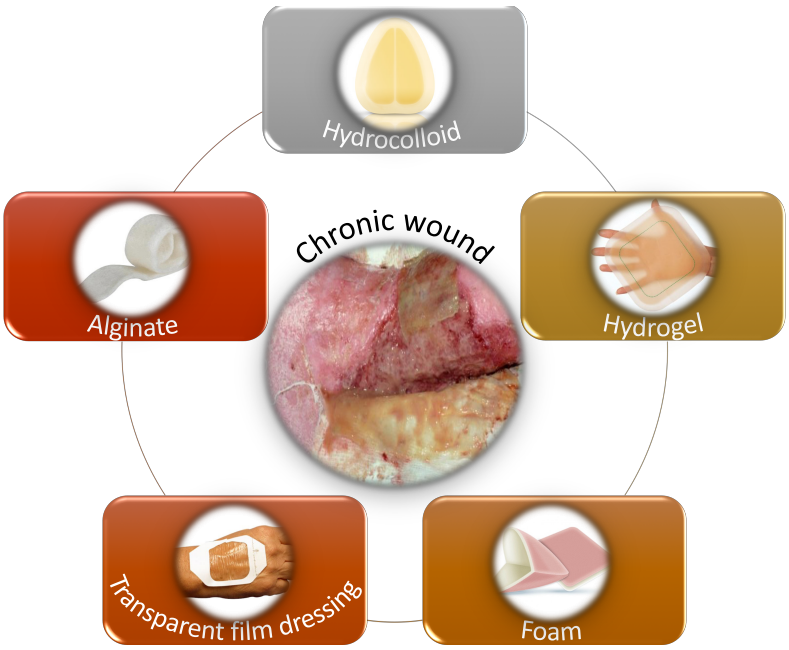

Figure 1.7. Example of common type of wound dressings, used for treatment of chronic ulcers. Each dressing is appropriate for different type of wounds or different stage of the wound chronicity, depending on the wound exudate produced from the wound.

1.2.3. Biophysical techniques

Along with the increased incidences of chronic wounds, the need for additional treatment beyond the standard care with wound dressings has brought about various new biophysical technologies. Several therapeutic biophysical technologies have been clinically examined and proved

improvement in wound healing rates. Nevertheless, their exact mechanism of action is still under investigation and more clinical trials are needed in order to improve their expoloitation characteristics and selection of the most appropriate technique in each case. Although still questionable, many studies regarding the efficiency of the biophysical techniques rely on the presumption that the heavy bacterial colonization in chronic wounds can be managed by using these technologies[113]. The biophysical technologies can be subdivided into four groups: ultrasound, which includes low frequency ultrasound and extracorporeal shock wave therapy (ESWT), negative pressure wound therapy, phototherapy and electrical simulation, including conductively and inductively coupled methods.

Ultrasound, which is defined as the sound waves with frequencies over 20 kHz has been widely reported to alter the growth of bacteria both in planktonic or biofilm physical states[114–116]. Low-frequency ultrasound (LFUS), which spans the range 20-60 kHz preferentially decreases bacterial counts, removes necrotic tissue and minimizes blood loss. LFUS was reported to decrease exudate and fibrin slough, to reduce the wound size and increase the rates of wound closure, to decrease pain compared to mechanical and surgical sharp debridement, to disperse biofilms rendering bacteria more susceptible to antibiotics and immune clearance[117]. Extracorporeal shockwave therapy (ESWT), originally applied for kidney stone fragmentation is another type of acoustic energy-based technique applied in chronic wounds. Preclinical and clinical research indicated that ESWT stimulates the expression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase, inducing angiogenesis in wound tissue which improves the healing rates[118].

Negative pressure wound therapy, relies on application of mechanical forces which results in microdeformations caused by suction devices with an interface material, most commonly open-cell polyurethane foam. The 3D structure of the foam allows even distribution of the vacuum throughout the foam, which in turn improves the fluid drainage. The underlying mechanisms, responsible for the wound healing improvement are classified as primary and their corresponding secondary effects. The following four mechanisms were described as a primary effects: 1) macrodeformation or wound shrinkage; 2) microdeformation at the inteface wound surface-foam; 3) fluid drainage and 4) wound environment stabilization. Secondary effects include modulation

of inflammation, cellular proliferation, migration and differentiation, angiogenesis and granulation tissue formation, peripheral nerve response and alterations in bioburden. Nevertheless, further high-level clinical studies are needed to explore deeper the mechanism of action, the specific interface coatings and instillation therapy[119].

Phototherapy by using ultraviolet light C (UVC) (200-290 nm) which demonstrated bactericidal effect have been applied for treatment of chronic wounds, infected with methicilin-resistant *Staphylococcus aureus* in non-randomized study of three patients. However, the authors pointed out the potential risk of skin cancer development to highly intensive UVC exposure. Later, the effect of the UVC was tested *in vitro* by direct exposure or indirectly by using transparent plastic filter of clinically relevant bacterial strains. Positive bactericidal results were achieved by the direct method, however no killing effect was found when UVC was filtered through a transparent plastic sheet. This new type of therapy have shown beneficial outcomes both *in vitro* and in all examined patients, nevertheless more exaustive randomized studies are still needed to confirm its efficacy[120,121].

Electrical stimulation therapy is adjunctive therapy which deliver low level currency to the wound and periwound area. For this purpose, two or more oppositely charged electrodes, made of various materials are positioned on the surface of tissues. The applied current can be either direct or pulsed and a range of parameters, such as frequency, amplitude and duration can be chosen to supply electrical stimuli to the wound. It was hypothesized that the enhanced wound healing rates by application of electrical stimulation therapy can be assigned to the inhibtion of bacterial growth. In a study, two out of four types of electrical stimulation examined for their inhibitory effect on bacterial growth have shown positive results – continuous microamperage direct current and high voltage pulsed current[122]. In another study, antibacterial effects of electrical stimulation therapy were found on all tested gram-positive and gram-negative bacterial strains, where the effect of positive polarity was higher than that of negative polarity. However, the antibacterial effect was much lower compared to that of wound antiseptics[123].

1.3. Advanced treatment solutions

Although the available biophysical wound management techniques are attracting more attention during the last decade as adjunctive wound healing therapies, additional clinical trials are needed to draw clearer conclusion for their effectiveness in each particular case of chronic wounds. Wound dressings, which were designed for targeting single factor from the gamut of detrimental chronic wound factors is unsatisfactory, since the persistence of other important factors remains intact. Advanced wound dressings available nowadays on-market were shown in many cases to resolve problematic hard-to-heal wounds, however 'universal' dressing which is able to address multiple factors, common in most chronic wounds is still missing. Therefore the establishment of 'universal' dressing able to fulfill the requirements for effective wound therapy is still on-market necessity. Meanwhile, the recent advances in theoretical basics of acute and chronic wound healing arouse in demands for new wound dressings, which combine as much as possible beneficial properties in one material in order to enhance the effectiveness of wound healing therapies[124]. Numerous synthetic and natural polymers have been exploited for obtaining such materials able to create environment, beneficial for the wound healing properties.

1.3.1. Polymers for chronic wounds

The growing demands for efficient chronic wound therapies lead to implementation of different strategies related with the destructive for the tissues chronic wound microenvironment. Among the polymeric materials, synthetic and natural polymers both has their advantages and disadvantages for creating appropriate environment to aid progressing the wound healing process in the correct direction. Synthetic materials are characterized with high reproducibility and can be easily tuned and in many cases possess superior mechanical properties compared to their natural counterparts. However, synthetic polymers can provoke undesired immunological response and can impart toxicity to the surrounding cells. In contrast, natural materials are less toxic and can rarely cause immunological reaction. Important disadvantage of natural polymers which is yet to be overcome is their poor mechanical properties and lower reproducibility compared to their synthetically derived counterparts.

1.3.1.1. Synthetic polymers

During the last decades, wound dressings underwent huge improvement, and nowadays dressings for wound management are mainly made of synthetic polymers. Synthetic polymers, which were widely investigated for their potential application as wound dressings for healing of acute and chronic wounds include polyurethane (PU), poly(caprolactone) (PCL), poly(vinylpyrrolidone) (PVP), polyglycolyc acid (PGA), polyacrylic acid (PAA), polylactic acid (PLA), poly-D,L-lactide-co-D-glycolide (PLGA).

PLA can be prepared in different forms, such as membranes or electrospun nanofibers. The more developed area for producing PLA-based wound dressings is by electrospinning, where the requirement is using high molecular weight PLA (around or more 100000 g/mol)[125]. Due to its biocompatibility and biodegradability, PLA nanofibers are very suitable for covering and healing of wounds. For instance, it was found that local decrease of pH due to the PLA degradation product lactic acid had antibacterial effect and promoted epithelialization of the affected area[126]. Combination of PLA with PGA, which gives their co-polymer PLGA was found promising for wound healing applications due to its biocompatibility, mechanical strength and ease of manipulation into various shapes and sizes. The rate of degradation of PLGA-based materials is controllable and can be tuned to a desired values which has found PLGA implication in skin substitutes[127]. Polyurethanes (PUs) are synthetic polymers, which are build by urethane linkages in their main chain. PUs are synthesized by polyaddition polymerization of isocyanates and polyols. It is a widely used polymer for wound dressings and is generally found in the form of a soft foam. PU acts as semi-permeable membrane and protects the wound from the environment and bacterial invasion. Limitation, related to the PU foams is their relatively high adherence, which disadvantage can be overcome by adding collagen meshwork.

1.3.1.2. Natural polymers

The fabrication of naturally derived-based wound dressing materials is increasingly growing due to their relatively lower immunological reaction and cytotoxicity via human cells compared to the synthetic counterparts. The ability of natural polymers to mimic the ECM mechanics rendered them a materials of choice for guiding cell-ECM mechanobiology[128]. Wide range of natural

polymers, including chitosan (CS), hyaluronic acid (HA), dextran, cellulose, collagen, gelatin, silk fibroin, elastin, alginate and fibrin have been utilized for the preparation of wound dressings. Particularly, CS and HA for which is widely known to possess beneficial properties in wound healing can be used in its pristine form or can be further modified by introducing functional groups in order to enhance their *in vivo* performance.

1.3.1.2.1. Chitosan

Chitin is the second most abundant biopolymer after cellulose and is composed of N-acetylglucosamine repeating unit. Chitin can be found in nature as ordered crystalline microfibrils in the cell wall of fungi or yeast or in the outer shell of arthropods. The main source of chitin is the shell of crustaceans, such as crabs and shrimps. Chitin have been reported to possess beneficial properties for biomedical applications, however the poor solubility in water and common organic sovents rendered chitin difficult to process and hence limited its usefullness[129].

Chitosan is the most important derivative of chitin, derived by partial deacetylation under alkaline conditions (concentrated NaOH) or enzymatically in the presence of chitin deacetylase[129]. It is composed of randomly distributed N-acetylglucosamine and D-glucosamine residues, and their relative proportion, which can be expressed in percentage degree of deacetylation (% DD), determines its physicochemical and biological properties, relevant for its application. It is widely accepted that transition of chitin to chitosan occurs when the DD>50 %.

Contrastingly to its precursor chitin, chitosan is soluble in aqueous acidic media, which property occur due to the protonation of the NH_2-groups on the C-2 of the polymer backbone. The chitosan gel-forming capability, high absorption capacity, biodegradability, biocompatibility and non-toxicity to living tissues and its antibacterial, antifungal and antitumoral properties renders it as a valuable material for number of biomedical applications. The most important branches from the biomedical field are tissue engineering of bone, cartilage, tendon and ligament, skin, nerve and liver, drug and growth factor delivery and wound healing where chitosan can be easily processed in different forms according to the requirements for its specific application[130–134].

Important issue in using polymers for biomedical applications is their metabolic turnover and biodegradation. The most common way of polymer degradation *in vivo* is by enzymes, able to attack specific bonds in the polymer backbone. Several enzymes, present in the human organism have shown activity towards chitosan degradation, such as lysozyme, bacterial enzymes in the colon and chitinases. Chitosan has been efficiently degraded *in vitro* by lysozyme and the degradation rate was dependent on the degree of deacetylation, where chitosan with lower degree of deacetylation (more chitin like) have shown faster degradation profile. Lysozyme is an antibacterial enzyme which is abundant in various human body fluids, such as saliva, tears, serum and gastric juice. It was demonstrated, that lysozyme can serve as a marker of infection in wound fluids[135]. Lysozyme attacks the β-(1,4) linkages between N-acetylglucosamine and N-acetylmuramic acid in peptidoglycan from the cell wall of different bacteria, leading to bacterial lysis. Similarly to peptidoglycan, chitosan possess β-(1,4) bonds, rendering it susceptible to cleavage by lysozyme. Despite of this susceptibility, it was shown that modifications such as crosslinking and thiolation significantly can alter chitosan degradation profile.

The antibacterial properties of chitosan with different physicochemical characterteristics were demonstrated and assessed in many studies, where modified or non-modified chitosans revealed antibacterial propeties against wide range of microorganisms[136–141]. The destructive for bacteria mechanism of action is still under investigation, nevertheless the most acceptable hypothesis relies on the interaction between the positively charged chitosan molecule and the peptidoglycan from bacteria cell wall. Chitosan is most active at the fungi and bacteria cell surface leading to permeabilization. The overall cationic character of chitosan stems from the presence of multiple amino groups, which in acidic environment can be easily protonated to give NH_3^+. It was postulated, that the electrostatic interactions between the protonated amino groups and the negatively charged peptidoglycan leads to the peptidoglycan hydrolysis and cell lysis with subsequent leakage of essential bacterial components and ultimately results in bacterial death. The most important factors towards the mode of chitosan action are the type of microorganism, the molecular weight and the degree of deacetylation[140].

One of the most researched biomedical fields, where chitosan-based materials have been applied is wound care owing to its multiple positive effects on wound healing. Chitosan has effect on PMNs by enhacning the production of osteopontin and increase of the complement activity, on macrophages by inducing the expression of activation markers, and biological mediators such as inteleukin-1 (IL-1), transforming growth factor (TGF)-ß1 and platelet derived growth factor (PDGF) and on fibroblasts by production of IL-8[142]. Chitosan can be applied onto the wound surface in different forms, such as sponges, powders and films which turn into a hydrogels upon hydration by the wound fluid. Chitosan film, loaded with basic fibroblast growth factor (bFGF) was tested on a wound incision of genetically-induced diabetic mice. The wound was characterized with accelerated angiogenesis and granulation tissue formation and the authors concluded that the bFGF-chitosan film can be used for treatment of chronic wounds[143]. In another study, antibiotics such as vancomycin and amikacin were absorbed on a porous chitosan sponge over 72 h. The *in vitro* antibiotic elution profile revealed that the sponge can be used as a drug carrier for treatment and protection against wound infection[144].

1.3.1.2.2. Thiolated chitosan

Chitosan functional groups offer many possibilities for its modification. The main target groups for modification are the hydroxylic group in C-6 position and the NH_2 group. Modification of chitosan aims at improving its physicochemical and bioactive features for various biomedical applications, without altering the chitosan backbone structure in order to preserve the main chitosan features. Amongst the synthesized chtiosan deriavtives, significant interest has gained trimethyl chitosan, N,N-carboxymethyl chitosan, O-carboxymethyl-N,N,N-trimethyl chitosan as a result in the improved chitosan solubility, antibacterial activity, ability to complex DNA and drugs and biocompatibility[145,146]. Another way to improve the functional characteristics of chitosan is by introducing thiol groups. Various thiolating agents can be immobilized onto the chitosan backbone, such as cysteine, thioglycolic acid (TGA), 2-iminothiolane hydrochloride, mercaptonicotinic acid (MNA) through the highly reactive amino group. One useful property recently achieved by functionalization of chitosan with thiol groups is the mucoadhesiveness.

Thiolated chitosan is able to form disulfide bridges with cysteine-rich domains of mucus glycoproteins, resulting in significant of up to 140-fold improved mucoadhesion, compared to that of unmodified chitosan. Mucoadhesion assure the localization of drug delivery systems at a given target site, and in situ permeation of drugs can be enhanced. In a study, conducted by Krauland et al., it was demonstrated that the bioavailability of chitosan-based vehicle, loaded with insulin via nasal administration was greatly enhanced for a thiolated chitosan carrier compared to this for an unmodified chitosan.

From the other hand, thiol functionalities give the possibility for disulfide bridge formation upon oxidation, which provides injectable properties and in addition can improve the mechanical properties of chitosan-based hydrogels. It was demonstrated that the elastic properties were improved by increasing the amount of thiol groups. Krauland et al. showed that in situ formation of hydrogels can be achieved by inter and/or intramolecular disulfide bonds. Controlled release of fluorescent dextran was optimized by varying the degree of crosslinking[147]. Thiols as strong electrophiles can easily react with acrylates, vinyl sulfones or oxidized polyphenols through Michael –addition reaction. In this way were prepared in situ forming chitosan-based hydrogels for drug delivery applications[148].

It was demonstrated that chitosan stability towards lysozyme degradation can be improved by modification of chitosan through thiol groups. Thiolated chitosan in solution revealed lower degradation rates, in case higher amount of thiol groups were immobilized. Moreover, when thiolated chitosan was subjected to crosslinking by air-oxidation of thiol groups to disulfide bridges, the degradation rate was exteneded[149]. Later, it was demonstrated that the chitosan degradation rates depended on the type of thiol bearing ligand immobilized on chitosan, the diversity and concentrations of the enzyme. Slower degradation of chitosan was observed when the thiolating agent was with short aliphatic chain, such as TGA. Contrastingly, when the thiolating agent was bulk aromatic molecule, such as MNA the degradation rates were faster compared even to pristine chitosan[150].

Thiol-containing cellular components, such as glutathione (GSH), thioredoxin, lipoic acid and metallothioneins can act as antioxidants by formation of disulfide bridges in presence of oxidative

environment[151]. Similarly to the natural thiol-bearing cellular antioxidants, functionalization of materials with thiol groups can improve their antioxidant properties. In this way, five-fold higher antioxidant activity was achieved when chitosan was functionalized with 2-iminothiolane (Traut's reagent)[152]. Thiol groups were found also to inhibit metalloenzymes, coordinating the metal ion from their actve center[153], which property can be exploited towards inhibition of Zn^{2+}-bearing MMPs.

1.3.2. Materials for single-factor directed therapies in chronic wounds

Materials, targeting various single factors in chronic wounds, have been synthesized to address the biochemical burden and aid in recovering the proper wound healing. Factors, which were with significant contribution for wound chronification are ROS, the bioburden, including gram-positive and gram-negative bacterial strains and deleterious wound enzymes, including MMPs and MPO. Targeting these factors can mitigate the wound chronicity, switching off the persistent inflammatory phase and direct the wound towards the proliferative phase. Many materials which are otherwise appropriate for exploitation as wound dressings lack of properties able to address most of the chronic wound constituents. In order to improve their performance in the chronic wound millieu, materials should be functionalized.

1.3.2.1. Antioxidant materials

Since the important role of ROS in the development of many pathological conditions have been demonstrated in many studies, the interest in creating materials with antioxidant properties, aiming at minimizing the detrminetal oxidative stress has considerably grown. This lead to the synthesis of materials, which can either directly act on ROS by scavenging them, or materials that can display ROS-induced solubility switch or degradation[154]. The final goal under application of these materials is to prevent the cells from further persisting oxidative stress.

Materials, which are able to undergo solubility switch in presence of ROS, such as H_2O_2 have been used as drug delivery systems. In this way, hydrophobic-to-hydrophilic transition of polypropylene sulfide to the corresponding sulfoxide or sulfone and of selenium-containing block

copolymers to the corresponding selenoxides and selenones rendered these materials promising for drug delivery in oxidative environment. Degradation of materials upon exposure to ROS is another property which was utilized for drug delivery. For this purpose, materials which possess linkages, susceptible to oxidation by ROS, such as boronic esters, Si-C covalent bonding, proline oligomers or polythioketals were synthesized for various biomedical applications, such as imaging agents, anticancer drugs and for treatment of gastrointestinal diseases[154].

Application of materials able to respond to ROS is a strategy, recently gaining interest in the area of chronic wound healing. Decreasing the elevated levels of ROS by delivering antioxidant materials is promising strategy that can address the highly oxidative environment in chronic wounds. This will minimize the oxidation of susceptible biomolecules to ROS attack. Following this strategy, carboxymethylcellulose/propylene glycol hydrogels were enriched with antioxidant fern tannin extracts. The materials exhibited good healing rates, when applied onto wounds of diabetes-induced rats[155]. In another study the antioxidant properties of materials commonly used as wound dressings, such as carboxymethylcellulose, low (300 kDa) and high (3-6 MDa) molecular weight HA and benzyl esterified HA were assessed. All materials, except the low molecular weight HA revealed antioxidant properties via superoxide and hydroxyl radical species from polymorphonuclear leucocytes-derived ROS[156].

1.3.2.2. Antibacterial materials

Since the invention of penicillin in 1928, the battle with bacterial infections by using antibiotics has significantly improved the quality of medical care and have saved millions of human lives. The mechanism of antibiotic action is based on targeting either the protein synthesis, DNA replication or cell wall synthesis. However, the bacterial protective machinery lead to the development of multidrug resistant microorganisms which is the reason for the more frequent failure of antibiotic therapies. Thus, the demands for adequate treatment of multidrug resistant microorganisms has evolved in establishment of macromolecular antibacterial agents, which are less prone to resistance. The mechanism of action of these macromolecular bioactives is based on disruption of the bacterial cell membrane, leading to irretrievable damage, followed by the

leakage of cytoplasm elements and ultimately cell death. Antibacterial materials can appear in different forms, such as hydrogels, foams or electrospun nanofibers. Among them, hydrogels has attracted considerable interest, due to their ease of fabrication, biocompatibility and tunable mechanical properties to match soft and hard tissue mechanics[157–160]. As for many biomedical fields, antimicrobial hydrogels can be applied on acute or chronic wounds, in order either to prevent from wound infection or eradicate already formed bacterial consortium. Numerous antimicrobial hydrogels from synthetic or natural polymers has been synthesized and revealed broad spectrum antibacterial activity against gram-positive and gram-negative strains. Antimicrobial hydrogels can be subdivided in three subcategories, according to the classification of hydrogel matrices and antibacterial agents: 1) hydrogels containing inorganic nanoparticles; 2) hydrogels containing antibacterial agent and 3) inherently antibacterial hydrogels. The first group refers to hydrogels, loaded with NPs, which are released from the hydrogel matrix to the wound bed through a difussion principle. The rate of nanoparticle release from the hydrogel matrix is directly related to the antibacteral performance, since insufficient concentrations may hamper the antibacterial effect of the NPs. Some hydrogels loaded with metal NPs can exhibit stronger antibacterial effect only after light irradiation. For instance carboxymethyl cellulose hydrogel, embeded with Ag/Ag@AgCl/ZnO hybrid nanostructures exhibited stronger antibacterial effect *in vitro* and accelerated wound healing in *in vivo* mice model. The antibacterial activity of the hydrogel system against E.coli (95.95 % killing effect) and S.aureus (98.49 %) was achieved by visible light enhancement of ROS formation[161]. In another work were prepared antifouling hydrogels for healing of infected diabetic chronic ulcers. The hydrogels which were prepared by mixing maleic acid-grafted dextran and thiolated chitosan with silver nanoparticles were able to trigger immune response through the upregulation of CD68+ and CD3+ expression levels[162].

1.3.2.3. Materials, targeting wound enzymes

MMPs has served as a targets in a broad range of pathological conditions, such as osteoarthritis, periodontitis, inflammation, post-miocardial infarction remodelling, vascular and neurodegenerative diseases, tumor angiogenesis and metastasis and neuropsychiatric disorders.

In chronic wounds, the disturbed ratio MMPs/TIMPs in favor of MMPs leads to excessive destruction of ECM collagen, pro-healing factors and cell surface receptors. This elevated activity and uncontrolled degradation of important for the wound healing process factors leads to persisting inflammatory phase aggravating the skin integrity reconstruction. Reducing the activity of overexpressed MMPs was shown to improve the outcome of chronic wounds with different etiologies. Searching for molecules and combination thereof between molecules and materials able to mimic and retrieve the function of the natural MMPs inhibiting system lead to the establishment of different strategies for accomplishing this goal. The last years research was focused on the design of MMPs inhibitors, containing chemical groups which are able to chelate the Zn^{2+} ions from their active center.

Wound dressings, acting in two different principles have been designed for reduction of MMPs activity in chronic wound milieu: 1) superabsorbent dressings, by absorbing and retaining the excess exudate which contains elevated levels of MMPs[163,164] or 2) by engaging the MMPs in degradation of dressing made of natural MMPs substrates, i.e. collagen matrices and in this way deviating their activity from ECM degradation[165].

1.4. Crosslinking of biopolymers – hydrogel formation

During the last several decades, hydrogels gained significant interest in various biomedical branches, including oncology, cardiology, immunology, wound healing and pain management. Hydrogels are networks with 3D structure, formed by crosslinking of polymers most commonly in aqueous media. Due to their hydrophilic nature, hydrogels can retain huge amounts of liquids in their porous structure, in some particular cases up to 1000 times of their own weight. Key physicochemical characteristics, such as mesh size and degree of crosslinking of hydrogels can be controlled by varying the concentrations of the polymer and the crosslinker. The crosslinked polymer structure render hydrogel solid-like and hydrogels with wide range of mechanical properties can be fabricated. For instance, their stiffness can be tuned in the range 0.5 kPa – 5 MPa to match the mechanical properties of soft and hard tissues[166]. A range of chemical crosslinkers, such as carbodiimide, glutaraldehyde, ethyleneglycol diglycidylether or isocyanate

have been utilized for the preparation of crosslinked hydrogels with appreciable mechanical properties. However, most of these crosslinkers are harmful for the macroorganism and may impede further implication of hydrogels with otherwise valuable properties. Therefore, alternative methods for obtaining crosslinked hydrogel structures has raised to match the requirements for biomedical applications. For instance, the natural compound genipin was used to replace harmful crosslinkers for stabilizing collagen[167] or chitosan[168] materials. Avoiding the usage of harmful crosslinkers, can be achieved by using, i.e. enzymatic tools which upon their action contribute directly by catalyzing the crosslinking of the polymer chains or indirectly by activating a small molecule which thereupon serves as a crosslinker between the polymer chains. Special emphasis is considered in design of hydrogels for wound healing applications as hydrogels fulfill many of the requirements for 'ideal' wound dressings.

1.4.1. Enzymatic tools for crosslinking

Enzymatic reactions are often preferred over their chemical counteparts. Enzymes, usually applied in small amounts offer mild conditions for crosslinking of biopolymers, which in many cases can play beneficial role for preserving the original polymer backbone structure, compared to the harsh conditions, required for chemical crosslinking. Moreover, the final product is free of potentially toxic residual chemicals, improving the biocompatibility. Hence, enzymatically-assisted synthesis of hydrogels can be considered as highly beneficial method for establishment of materials for biomedical applications.

A rich arsenal of enzymes, classified in six big groups (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases) are nowadays being utilized for catalyzing a number of chemical reaction, which otherwise from thermodynamical point of view are difficult or even impossible to achieve. Up-to-date, transferases, hydrolases or oxidoreductases have been utilized for crosslinking of polymers or biopolymers. Amongst the transferases, transglutaminases (TGs) have been used to crosslink biomolecules with proteinaceous structure, or synthetic polymers bearing the corresponding amino acid residues. TGs typically catalyzes pH-dependent transamidation of glutamine residues to lysine residues[169]. Following the TG catalytic mechanism,

crosslinked hydrogels from end-modified polyethylene glycol (PEG) macromer and a synthetic polypeptide were prepared[170]. In another study, Zhao et al. prepared novel injectable smart crosslinked human-like collagen hydrogels, sensitive to temperature and enzymes. They used microbial TG to employ crosslinks between lysine and glutamine residues and envisaged potential application for skin tissue engineering of the obtained material[171].

Oxidoreductases, such as horseradish peroxidase (HRP), tyrosinase and laccase are able to catalyze the oxidation of phenol substrates to quinones. The oxidized quinone products are highly reactive towards amino and thiol nucleophiles, through Michael-addition reaction or Schiff base formation. Over these three oxidoreductases, laccases possess some advantadges and thus are preferred as green catalysts in biotechnological oxidative applications, such as bioremediation[172], organic symthesis[173–175], fiber modification/bleaching[176,177]. Laccase has very broad polyphenol substrate range, compared to tyrosinase which is more specific enzyme and catalyzes the oxidation of tyrosyl residues in protein molecules. Laccase is a copper-containing enzyme, which can catalyze mono-, di- and polyphenols, methoxyphenols, aminophenols, aromatic amines, hydroxyindols and benzenethiols single electron oxidation to the corresponding radicals[174]. From the other hand, laccase uses molecular oxygen as a final electron acceptor for its oxidation activity and releases water as the only by-product, while HRP needs hydrogen peroxide to perform its reaction of oxidation. In line with this important environmental and cost-effective issue, laccases attracted considerable interest for various applications in large scale industrial biotransformations. In order to enhance their capacitance for industrial biotransformations, laccases can be subjected to engineering by either directed molecular evolution or rational design with significant improvement of their stability in the desired media and conditions[178]. Disadvantage, which researchers face when working with laccase is its relatively low redox potential impeding the oxidation of substrates with higher redox potential. Nevertheless, solution to this shortcoming when using substrates with higher redox potential was found by using mediators and enhancers of laccase catalytic activity[179,180].

1.5. Wound dressings on market

It was estimated that during the year 2009, the global wound dressing market was worth 5.1 billion dollars. It is expected that the market will reach 17.3 billion dollars by 2023, according to P&S Market Research. This expenditure includes 234 million surgical interventions and different chronic wounds, such as 40-50 million leg ulcers, 35-40 million pressure sores and equal number of burn wounds. WHO estimated that the worldwide elderly population aged 60 or over in the more developed regions was 12 % in 1950, rose to 23 % in 2013 and is expected to reach 32 % in 2050. This rise in the elderly part of the population will inevitably lead to increase in non-communicable and neurological diseases and diabetes, which in most cases are the underlying pathology of different types of chronic wounds. As a consequence, this will cause aggravation to the health systems expenditures for treatment of various types of chronic wounds if not more efficient and cheaper option has been developed.

Several companies that dominate on the wound dressing market are offering products which can be used for treatment of acute or chronic wounds. The most common brands and their use in different types of wounds are summarized in table 1.

Table 1.1. Commercial dressings, offered on market from the big wound dressing manufacturers. Along with the type of dressing (alginate, foam, hydrogel or hydrocolloid) are given the correpsonding brand name and example of the most common use for each dressing.

Dressing	Example	Clinical application
Alginate	Algisite™ Algosterile® Kendall™ Kalginate® Kaltostat® Melgisorb® Seasorb® Sorbsan®	Deep and exudative pressure ulcers, pyoderma gangrenosum, diabetic wounds Bleeding wounds Donor sites
Foam	Allevyn®	Wounds over body prominences

	Aquacel®	Mildly exudative wounds
	Biatain®	Donor sites
	Biopatch®	
	Flexzan®	
	Kendall™ Curaform™	
	Kendal™ Hydrasorb®	
	Lyofoam®	
	Mepilex®	
	Polymem®	
Hydrocolloid	Duoderm®	Leg stasis ulcers
	Comfeel®	Arterial ulcers
	Cutinova®	Pressure ulcers
	Hydrocol®	Diabetic ulcers
	NuDerm®	Partial-thickness burns
	Replicare®	Donor sites
	Tegasorb™	Skin abrasions
		Superficial acute wounds
Hydrogel	Carrasyn®	Calciphylaxis
	Clearsite®	Coumadin necrosis
	Elasto-Gel™	Dry venous or arterial ulcers
	FlexiGel™	Painful, non-exudative wounds
	Hypergel®	
	Kendall™ Curafil™	
	Kendall™ Curagel™	
	Normlgel®	
	Nu-gel®	
	Tegagel™	
	Vigilon®	

Although the offered wound dressings has proven to possess beneficial effects in acute and chronic wound healing, no universally bioactive dressing which is able to address most of the detrimental chronic wound factors is available on market nowadays. Establishment of such

dressings and their on market exploitation will boost the endless battle with hard-to-heal ulcers in a more favorable pattern for patients, clinicians and healthcare systems.

2. Objectives of the thesis

Incidence of chronic wounds presents a serious global concern, which has dramatically increased during the last decades. The improved quality of life along with the demographic increase of the elderly population and associated cases of diabetes mellitus lead to prevalent incidences with chronic wounds. In the US alone diabetes affects 9.3 % of the population (29.1 million people) and 26 % of Americans over 65 years old. Patients with diabetes are 10 times more likely to require limb amputation during their lifetime, as a result of wound chronification. It was estimated, that 6.5 million in the US and 1.5-2 million in Europe suffer from chronic wounds[181,182]. Chronic wounds require dedicated medical care and huge costs for the health systems and in addition deteriorate the patient's quality of life, associated with pain, immobility, discomfort, depression and social isolation.

Despite the different origin of chronic wounds, they present one common feature - prolonged inflammation. The underlying molecular mechanisms, responsible for the persistent inflammation are the overexpressed proteolytic (matrix metalloproteinases) and oxidative (myeloperoxidase) enzymatic activities and the oxidative stress, due to excess production of reactive oxygen species (ROS). In addition, the skin-forming cells such as fibroblasts and keratinocytes are with reduced migration and proliferation capacity and are more prone to senescense, disturbing the process of normal wound healing. Finally, the continuous exposure of the wound to the environment, renders it more susceptible to heavy bacterial contamination and subsequent infection which can further worsen the morbidity.

The compexity of the wound healing process and multifactorial nature of chronic wounds rendered single-factor directed therapies low or non-effective. Therefore, **approaches which include the targeting of multiple factors** present in the chronic wound environment can enhance the healing rates, resulting in relieve of the detrimental outcomes and the economical burdens in treatment of chronic wounds.

The main objective of this thesis is to develop multifunctional biopolymer-based hydrogels based on the understanding of the molecular basics of non-healing (chronic) ulcers. The hydrogels, will be designed in such manner that several beneficial properties will be combined in a single material, in order to support the healing process in chronic wounds. The new dressings,

prepared from appropriately modified biopolymers and small organic natural bioactives will possess suitable physicochemical and bioactive characteristics to address multiple factors manifesting in chronic wounds.

The following specific objectives define the roadmap towards the development of efficient chronic wound dressing materials:

1) **To generate multifunctional biopolymer-based hydrogels covalently crosslinked with bioactive species.** Covalently crosslinked hydrogels will be generated in a one step environmentally friendly enzymatic reaction. For this purpose, an oxidative enzyme (laccase) will be used to oxidize natural phenolic compounds, which consequently will covalently crosslink the previously modified (thiolated) biopolymer (chitosan) to form the hydrogels. Besides their function as crosslinking agents, the polyphenolic molecules will provide the hydrogel platform with additional bioactive features, such as antioxidant and antimicrobial, and inhibitory activity over wound myeloperoxidase.
2) **To characterize the structural and rheological properties of the produced hydrogels**. The pore size of the hydrogels, their rheological behaviour along with the chemical structure will be determined.
3) **To determine the physicochemical properties of the new hydrogels in physiological conditions.** The acceptance of materials as wound dressings largely depends on their ability to appropriately interact with the wound environment. The swelling properties of the hydrogels, their stability in presence of enzymes overexpressed in chronic wounds will be assessed accordingly for evaluating the applicability of the novel materials as wound dressings.
4) **To evaluate *in vitro* the bioactivities of the hydrogels against major factors governing wound chronicity and in presence of specific human cells**. The bioactive properties of the hydrogels will be assessed in order to validate their potential application as dressings for chronic wounds. A balance between their inhibitory capacity against major chronic wound enzymes, their antioxidant and their antibacterial activity from one hand and their

biocompatibility via cell lines necessary for the healing process from another hand should be achieved.

5) **To assess *ex vivo* the bioactivities of the hydrogels against major chronic wound enzymes in a real wound fluid.** The bioactivity performance of the hydrogels will be assessed in a real wound exudate, which contains major chronic wound enzymes, i.e. myeloperoxidase and total matrix metalloproteinases. Testing hydrogels performance in wound fluid extracted from patient with chronic wound will allow for their evaluation in an environment which represents the real biochemical mileu of the chronic wound. Their *ex vivo* inhibitory capacity will serve as a prerequisite for further *in vivo* evaluation.

3. Summary of the main results

In this section a summary of the most significant published results of this thesis is presented. Overall, the results revealed that thiolated chitosan based hydrogels are promising candidates for application as multifunctional bioactive wound dressings for treatment of chronic wounds. In Papers I and II it is shown, that thiolated chitosan gelates through enzymatically-mediated reaction by using naturally-based polyphenols. Laccase oxidizes polyphenols to highly reactive quinones, which further undergo Michael-addition and Schiff base reaction toward nucleophiles, such as amino and thiol groups acting as crosslinkers of the biopolymer chains. The results in Papers I and II, demonstrate the suitability of the hydorgels as a multifunctional platform for treatment of chronic wounds addressing most of the detrimental chronicity factors, such as elevated levels of MMPs, MPO and ROS, and bacterial contamination.

3.1. Paper 1

Multifunctional Enzymatically Generated Hydrogels for Chronic Wound Application

Ivaylo Stefanov, Sílvia Pérez-Rafael, Javier Hoyo, Jonathan Cailloux, Orlando O. Santana Pérez

The aim of this study is to sythesize multifunctional hydrogels as a bioactive platfroms for treatment of chronic wounds. For this purpose, thiolated chitosan with different degree of thiolation is used as a matrix and crosslinked with small naturrally-based polyphenol gallic acid acting as a crosslinker and bioactive compound. Gallic acid is oxidized in situ in an environmentally-friendly reaction by using oxidoreductive enzyme laccase to the highly reactive quinones, which further undergo reactions with nucleophiles, such as thiol (-SH) and amino ($-NH_2$) groups.

In this paper it was elucidated, that the oxidized gallic acid reacts predominantly with the stronger nucleophilic thiol groups, and hydrogels were not yielded when pristine (non-modified) chitosan with solely amino groups, was used. The chemistry of the enzymatically-assisted reaction was elucidated with FTIR-spectroscopy where the characteristic wavenumber corresponding to the -SH band disappeared, confirming its consumption during the hydrogel formation. Time sweep

rheological measurements reveal the strong influence of the thiol groups on the gelation process, where the thiolated chitosan with higher amount of thiol groups gelated significantly faster than those with lower amount. Results show that hydrogels possess multifunctional properties relevant for their application as platforms for treatment of chronic wounds, including their ability to inhibit deleteriuos chronic wound enzymes, such as MMPs and MPO, to scavenge ROS and to inhibit the growth of the most frequently isolated from chronic wounds Gram-positive and Gram-negative bacterial species. It is demonstrated that the thiolated chitosan, which is crosslinked with higher amount of gallic acid exhibit higher *ex vivo* inhibitory activity against both MMPs and MPO. The overall results lead to the conclusion that these hydrogel platforms can be exploited as efficient multifunctional bioactive dressings.

3.2. Paper 2

Enzymatic synthesis of a thiolated chitosan-based wound dressing crosslinked with chicoric acid

Ivaylo Stefanov, Dolores Hinojosa-Caballero, Santiago Maspoch, Javier Hoyo, Tzanko Tzanov

The aim of this study is to synthesize multiunctional bioactive platforms for treatment of chronic wounds. Herein, the short and structurally rigid gallic acid used in Paper I is replaced by another polyphenol chicoric acid. It possess two polyphenol aromatic cores, connected by eight-carbon chain resembling the chemical structure of commonly used homobifunctional crosslinkers. The results revealed that upon laccase oxidation, chicoric acid successfully crosslinked chitosan chains through thiol Michael-addition reaction, confirmed by Raman spectroscopy. Morphological characterization by using SEM, reveal smaller pore size and more homogenous structure in addition to the greater mechanical properties compared to the hydrogels generated in paper I. The last results are achieved by using concentration of chicoric acid in the micromolar range, while the concentration of gallic acid in Paper I was in the millimolar range. This envisages the effectiveness of chicoric acid in enzymatically-assisted crosslinking of other thiomers. The performance of the hydrogels via inhibition of deleterious chronic wound enzymes, scavenging

ROS and growth inhibition of clinically relevant Gram-positive and Gram-negative bacterial species confirms the multifunctional properties of the hydrogels in healing of chronic wounds.

4. Main conclusions and future plans

4.1. Main conclusions

Incidences with chronic wounds has considerably increased the last years, due to the demographic increment of the elderly fraction of the world's population. The necessity for well-established programmes and well-educated clinicians with their integration into hospital units, increased the possibility for positive outcome in treatment of patients with chronic wounds. Nevertheless, the lack of universal and effective dressings is still challenging and the demands for such dressing are annually rising. Understanding the complex chronic wound milieu has brought about the development of different approaches, which aim at targeting single factors, that are delaying the normal wound healing. However, since each factor has considerable contribution for the overall wound chronicity, single-factor oriented approaches hold little potential for positive outcomes in the management and healing of chronic wounds. Therefore development of bioactive dressings with panel of properties, able to address most of the factors, contributing for the wound chronicity is still necessary. Following the above, multifunctional hydrogel platforms for treatment of chronic wounds were developed during the implementation of this thesis. The following conclusions addressing the main objectives of the thesis can be drawn:

1. Hydrogels were synthesized using low molecular-weight thiolated chitosan as a matrix, which was crosslinked by the natural polyphenolic compounds gallic and chicoric acid, in situ oxidized by an environmentally friendly enzymatically-assisted reaction.
2. Thiol functionalization was crucial for hydrogel formation. The amount of thiol groups, immobilized on chitosan revealed significant impact on the hydrogels rheological profile, demonstrated by shorter gelation time and improved mechanical properties of the gels obtained from chitosan with higher amount of thiol groups. Furthermore, the type of polyphenolic acid used had also significant impact on hydrogel properties. Hydrogel prepared with chicoric acid have shown faster gelation time, even in lower than gallic acid concentration.

Therefore, chicoric acid can be used as a more effective naturally-based crosslinker for thiol Michael-addition based synthesis of hydrogels.

3. The biodegradability profiles of the hydrogels have shown that the hydrogels prepared with chicoric acid were more stable than those preared with gallic acid. This result translates into proplonged, up to 7 days, application on the wound site.
4. Hydrogels with tunable *ex vivo* inhibitory activity towards deleterious chronic wound enzymes, such as MMPs and MPO were produced. Modulation of the MMP/MPO inhibitory efficiency of the hydrogels was more feasible with hydrogels crosslinked with chicoric acid. This was due to the greater crosslinking efficiency achieved with lower concentrations of chicoric acid than gallic acid and hence more preserved thiol groups, acting as deleterious chronic wound enzyme inhibitors.
5. The antibacterial effect of the hydrogels against major gram-positive and gram-negative bacterial strains, frequently found in chronic wounds generally increased with decreasing the amount of thiol groups and amount of chicoric acid. This tendency was related to the higher amount of antibacterial amino groups in the case of hydrogels prepared by thiolated chitosan with less thiol groups.

4.2. Future plans

The results presented in this thesis, revealed that: 1) thiolated polymers (thiomers) and polyphenols can be easily gelated by a laccase-assisted reaction; 2) the novel hydrogel materials have shown bioacivities that can positively affect the outcome of chronic wound therapies, while their biodegradability profile have fulfilled the requirements for prolonged application on the wound site. The same enzymatically-assisted reaction pattern can be utilized for the synthesis of other thiomer-based hydrogels with potential application in chronic wound healing. Pre-clinical

validation of these dressing materials in animal models with induced chronic wounds will be a necessary step towards clinical applications. From the other hand, carbohydrates which does not intrinsically possess antibacterial properties like chitosan can be upgraded with, i.e. hybrid silver/polyphenol nanoparticles which, besides their antibacterial properties can serve also as centers for crosslinking. In this way, nanocomposite hydrogels with superior antibacterial properties and sustained release of the antibacterial compound can be synthesised for application on infected chronic wounds. For instance, hyaluronic acid which was proved to possess wound healing properties can be modified with thiol groups and further serve as matrix for the incorporation of antibacterial nanoparticles.

The high biocompatibility of the hydrogels against fibroblast skin cell lines revealed that the gels can be regarded not only as a materials for the treatment of chronic wounds, but also as a scaffolds for encapsulation of cells for therapeutic delivery. Therefore, the area of application of thiomer-based hydrogels can be expanded to treatment of pathological processes, which constitute similar deleteriuos species as the chronic wounds which can be eradicated by the hydrogel bioactives and at the same time should be treated with specific cell types.

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5. Paper I

Multifunctional Enzymatically Generated Hydrogels for Chronic Wound Application

Pages 85 to 96 of the thesis, containing the paper mentioned above can be found at the editor's web:
https://pubs.acs.org/doi/pdf/10.1021/acs.biomac.7b00111

6. Paper II

Enzymatic Synthesis of a Thiolated Chitosan-Based Wound Dressing Crosslinked with Chicoric Acid

Pages 99 to 109 of the thesis, containing the paper mentioned above can be found at the editor's web:
https://pubs.rsc.org/en/content/articlelanding/2018/tb/c8tb02483a - !divAbstract