Development of multifunctional biopolymeric materials for treatment of decubitus ulcers

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1965
SÍ, BUENO, TRABAJAR PARA GANARSE LA VIDA, CLARO
¿PERO POR QUÉ ESA VIDA QUE UNO SE GANA TIENE QUE DESPERDICIARLA EN TRABAJAR PARA GANARSE LA VIDA?
QUINO

Abstract

Chronic wounds including pressure, venous, arterial and diabetic neuropathic ulcers, represent a significant burden to the healthcare system. These different chronic wound types do not share origin or cause; however they have common features as bacterial infection and continuing influx of polymorphonuclear neutrophils that release high concentrations of matrix metalloproteases (MMPs), myeloperoxidase (MPO) and reactive oxidative species causing excessive degradation of the extracellular matrix (ECM) and the growth factors. Because of the multifactorial nature of virtually all chronic wounds, the therapeutic wound healing approach should emphasize the necessity to investigate wound dressings that possess the ability to directly and indirectly modulate the biochemical environment based on the pathology of chronic to encourage the healing process.

The overall aim of the present work was to develop biopolymer wound dressings capable to improve the management of chronic wounds. The specific research objective was defined in three main targets: i) to balance the proteolytic activity of MMPs, ii) to balance the MPO activity and the oxidative environment, and iii) antimicrobial protection.

The first part of the thesis aimed to provide suitable materials to perform bioactive biopolymer-based wound dressings. The first step was to provide a versatile functionalization of chitosan platform to improve the sequestering ability over metal dependent enzymes (MMPs) in chronic wounds. In order to impart to chitosan the ability to inhibit MMP, chitosan was functionalized with deferent thiol moieties, which can chelate the zinc cation in the active site of the enzyme, modifying MMP activity. In addition the combination of -NH_2 and -SH chemistry allowed grafting of other active agents on the chitosan platform.

The second step was the identification and evaluation of natural active agents as chronic wound healing promoters. Polyphenols from bark, twigs and leaf extracts from the medicinal plant *Hamamelis virginiana* (Witch hazel) were studied for this porpoise and it was found to have both a strong antioxidant activity and an inhibitory effect on MPO and collagenase.

The second part of the thesis was focused in the performance and evaluation of new materials for wound healing applications. To this aim, chitosan and/or thiolated chitosan were modified with *H. virginiana* extracts following different approaches.

Abstract

The first approach was focused to develop a new method for covalent functionalization of thiolated chitosan with polyphenols from *H. virginiana* extracts. The novelty of this approach consists in the use of thiolysis - a common analytical method for proanthocyanidins characterization - to covalently functionalize natural macromolecules such as chitosan with bioactive phenolic moieties. The phenolics-functionalized chitosan showed improved therapeutic properties *in vitro*.

The second approach focused on the use of laccase-assisted cross-linking between phenolic moieties of *H. virginiana* with chitosan and gelatin as a functionalization method to obtain stable and bioactive hydrogel wound dressings. *H. virginiana* extract was oxidized by laccase in a one-step process under mild reaction conditions to covalently crosslink chitosan and gelatin. The physical and mechanical properties of these hydrogels were investigated using different analytical techniques and their potential for chronic wound treatment was evaluated *in vitro* in terms of antibacterial and inhibitory effect on MPO and collagenase. The results indicated that the polyphenols exerted a dual role in the hydrogel: i) "passive" – being a structural element, and ii) "active" – modifying the chronic wound environment by attenuating the deleterious MMPs, MPO and ROS activities, and reducing the bacterial load.

KEYWORDS • Chronic wound • Wound dressing • Collagenase • Myeloperoxidase • Antimicrobial activity• Chitosan • Thiolated chitosan • Plant polyphenols • *Hamamelis virginiana* • Thiolysis • Laccase • Cross-linking • Hydrogels •

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Chapter 1

Wound dressings: State-of-the-art

1.1. Introduction

Throughout the centuries, mankind has applied a variety of readily available natural substances to wounds to staunch bleeding, absorb exudates and promote healing. These include honey, cobwebs, leaves and plant extracts, moss and animal dung. In the nineteenth century, primitive absorbent dressings were made by unpicking old rope (oakum) or rags in order to obtain a fibrous mass of flax or cotton fibres that could be used to pack cavities and soak up exudates or blood.

Simple absorbent dressings played a major role in wound management for many years gradually being improved by the addition of low-adherent wound contact layers made from perforated plastic films, nonwoven fabrics or other suitable materials.

Since acute wound healing follows a well-defined pattern resulting in complete wound closure it is possible to objectively assess its progression throughout the healing process. On the contrary, due to the inherent alterations in the pathophysiology of chronic wounds, the biological effects of ageing on the healing process and the direct confounding effect of associated co-morbidities in the elderly, the healing of chronic wounds is often protracted, variable and non-uniform [1].

Furthermore, in most clinical trials and studies involving wound dressings and devices, 'healing' is considered to be the primary predetermined endpoint for both acute and chronic wounds. However, products that are aimed to achieve healing in acute wounds may not have the same effect in chronic non-healing wounds due to the interaction of various confounding factors such as exudate, an unhealthy wound bed and infection.

Considerable progress has been made on advanced products in the field of wound healing and a number of new therapeutic approaches are now available. It is hoped that continued advances will come about which, when combined with basic medical and surgical approaches, will accelerate the healing of chronic wounds to an extent that is still not possible with current therapeutic agents

The science of wound healing is advancing rapidly, particularly as a result of new therapeutic approaches such as growth factors, skin substitutes, and gene and stem cell therapy.

1.2. New therapeutic approaches in wound healing

1.2.1. Gene therapy

The technology to introduce certain genes into wounds by a variety of physical means or biological vectors, including viruses, has existed for some time. These range from *ex vivo* approaches, where cells are manipulated before being re-introduced into the wound, to more direct *in vivo* techniques that may rely on a simple injection or the use of a gene gun [2, 3]. Gene therapy as a whole is a very active area of research, with 320 clinical protocols submitted to regulatory bodies around the world since 1999 [4].

1.2.2. Stem cell therapy

Extending the hypothesis that cell therapy may be required to recondition chronic wounds and accelerate their healing leads to the conclusion that stem cells may offer even greater advantages [5].

1.2.3. Bioengineered skin. Tissue-engineered 'skin equivalents'

A number of bioengineered skin products or skin equivalents have become available for the treatment of acute and chronic wounds as well as burns [6-8] become as widespread as desired.

Bioengineered skin may work by delivering living cells which are known as a 'smart material' because they are capable of adapting to their environment. There is evidence that some of these living constructs are able to release growth factors and cytokines [9, 10] but this cannot yet be interpreted as their mechanism of action. It should be noted that some of these allogeneic constructs do not survive for more than a few weeks when placed in a chronic wound [11, 12].

1.2.3.1. Cell-free matrices

Two approaches are currently used in the production of cell-free dermal matrices. The first is a synthetic matrix, usually comprising collagen and other extracellular matrix components, that attempts to recreate the desired physical and chemical properties of the dermis. The second approach is the use of native dermis, from which the cellular components have been removed. This may be treated to preserve the dermal architecture.

1.2.3.2. Cell-containing matrices

As with cell-free matrices, cell-containing matrices include both synthetic matrices, often made of polyglycolic acid mesh, as well as natural biological substrates usually comprising collagen and glycosaminoglycans. Alternatively, non-cellular matrices, such as the hyaluronic acid scaffolds, are recommended for culture with autologous patient cells prior to grafting.

1.2.4. Molecular Therapy

Therapeutic interventions with the potential to correct degraded ECM. These research finding have led to the development of several wound dressings with the aim of inactivating MMPs in chronic wounds [13-17] in combination with other therapies.

1.3. New medical dressings in wound healing. Factors influencing dressing development/selection.

Many sophisticated dressings are available to the wound care practitioner, made from a wide range of materials including polyurethane, salts of alginic acid and other gelable polysaccharides such as starch, carboxymethylcellulose and chitosan. These materials may be used alone or in combination to form products as diverse as films, foams, fibrous products, beads, hydrogels or adhesive gel-forming wafers more commonly called hydrocolloid dressings.

Depending upon their structure and composition, such dressings may variously be used to absorb exudate, combat odour and infection, relieve pain, promote autolytic debridement (wound cleansing) or provide and maintain a moist environment at the wound surface to facilitate the production of granulation tissue and the process of epithelialisation.

Some dressings simply absorb exudate or wound fluid and may therefore be suitable for application to a variety of different wound types. Others have a very clearly defined specialist function and as such have a more limited range of indications.

This may mean that they are only suitable for the treatment of specific types of wounds or for the management of a wider range of wounds during a particular phase of the healing cycle. Wound healing is a dynamic process and the performance requirements of a dressing can change as the wound progresses towards healing.

Occasionally, the pain associated with dressing changes can be such that a patient requires potent analgesia. In these circumstances the priority may be to identify a product that can be easily removed whilst causing minimal pain and trauma [18].

If clinical infection is present, it may be necessary to use a dressing that has proven antibacterial properties as an adjunct to systemic therapy.

1.3.1. Wound related factors

1.3.1.1. Wound Type

A simple wound classification system such as that shown below, forms a useful starting point in the selection process. Within this classification wounds are divided into four basic types according to their appearance but it will be immediately obvious that these groups could be further sub-divided to take account of wound aetiology or depth, for example.

1- *Necrotic wounds* - covered with devitalised epidermis, frequently black in colour,

2- *Sloughy wounds* - which contain a layer of viscous adherent slough, generally yellow in colour,

3- *Granulating wounds* - which contain significant amounts of highly vascularised granulation tissue, generally red or deep pink in colour,

4- *Epithelializing wounds* - which show evidence of a pink margin to the wound or isolated pink islands on the surface.

It will be recognised that these descriptions relate not only to different types of wounds but also to the various stages through which a single wound may pass as it heals.

1.3.1.2. Wound Infection

All wounds, regardless of type, may become colonised by microorganisms which in turn may cause the formation of unpleasant odours. If these organisms increase in number past a certain critical level, then the wound may develop a clinical infection [19] which may necessitate treatment with systemic antibiotics and/or the use of a medicated dressing. Sometimes a wound can become infected with an organism that is resistant to normal antimicrobial therapy and in these instances a dressing that forms an effective bacterial barrier can be used to cover the wound in order to prevent the spread of the resistant organism [20].

1.3.1.3. Wound Location

The location and size of the wound are also important factors. The location of the lesion may make application of a particular product almost impossible.

Wound related factors which have an influence upon dressing development may therefore be summarised as shown below. Any or all of these factors may determine the performance requirements of a dressing and thus have a marked influence on the development process.

Table 1.1. Factors influencing dressing development.

Wound type	superficial
	full thickness
	cavity
Wound description	necrotic
	sloughy
	granulating
	epithelializing
Wound characteristics	dry
	moist
	heavily exuding
	malodorous
	excessively painful
	difficult to dress
	liable to bleed easily
Bacterial profile	sterile
	colonised
	infected
	infected and potential source of serious cross infection

1.3.2. Product related factors

The major characteristics of a dressing that determine its suitability for application to a particular type of wound include its conformability, fluid and odour absorbing characteristics, handling and adhesive properties and the presence of antibacterial and haemostatic activity where appropriate. Other more general factors which may also influence product selection include the potential for the dressing to cause sensitivity reactions, the ease of application and removal (including the production of pain and trauma to wound surface) and the interval between dressing changes. Dressings should also not shed particles or fibres that may delay healing or predispose the wound to infection. They should also not contain extractables that may have an adverse effect of cell growth.

The product related factors may therefore be summarised as follows;

- conformability
- mass or volume (for cavity wounds)
- fluid handling properties
- sensitisation potential

- odour absorbing properties
- antibacterial activity
- haemostatic properties
- permeability to tissue fluid and microorganisms
- ease of use
- pain related factors
- fibre-fast
- non-toxic.

1.3.3. Patient related factors

The major patient related factors may be summarised as follows;

- wound aetiology,
- state of continence,
- known sensitivity to medicated dressings,
- fragile or easily damaged skin,
- the need to bathe or shower frequently.

1.3.4. Economic considerations

The principal economic considerations relate to cost and availability. These factors may therefore be summarised as follows;

Table 1.2: Economic factors

Cost	unit cost
	treatment cost
	cost of alternative materials
Availability	on prescription (Country Sanitary Service)
	in stores or pharmacy departments
	inclusion in local formularies

1.4 Wound dressings in the market

- Absorbent, perforated plastic film faced dressings

These dressing were probably the first serious attempt to improve on the cotton gauze 'dry dressing', and combine absorbency with reduced risk of adherence. They were also the first to be described as 'non-stick' or non adherent. Compared with the fabric dressings used before, however, they were certainly low adherent.

- Absorbent, perforated film dressing with adhesive border

***- Knitted viscose primary dressing BP*:** These dressings are designed to be used with a secondary dressing, depending on the level of absorbency required; they rely on the low adherent property of knitted viscose.

***- Povidone iodine fabric dressing*:** A knitted viscose primary dressing impregnated with povidone iodine

- Vapour permeable adhesive film dressing BP: Though they have found a variety of uses in securing primary dressings intravenous cannulae and other medical devices, their main indication is lightly exuding, clean, superficial wounds.

- Activated charcoal cloth with silver

- Alginate dressings: Possibly one of the most underrated of primary dressings, alginates have the supreme advantage over their competitors that the dressing can simply be soaked off with saline or a low-force shower; even after several days. Useful for medium to heavily exuding wounds (or parts of wounds), alginates are not the dressing of choice for infected wounds; there is little point in using alginates for dry wounds.

- Hydrocolloid dressings: Probably the first dressings to fully exploit the concept of moist wound healing; there is still a major role for hydrocolloid dressings in light to medium exuding wounds. Hydrocolloids are not ideal for infected wounds. The dressing requires a margin of smooth skin around the wound to ensure adhesion. Dressings need frequent change with heavily exuding wounds.

- Hydrogel dressings: Particularly useful for dry, sloughy or necrotic wounds, hydrogels have a role in lightly exuding wounds and granulating wounds. Hydrogels are not suitable for infected or heavily exuding wounds; an appropriate secondary dressing is required.

-Polyurethane foam dressing BP: Indicated for light to medium exuding wounds; not recommended for dry superficial wounds.

- Polyurethane foam/film dressings: Indicated for light to medium exuding wounds; not recommended for dry superficial wounds.

Table 1.3. Common wound healing products on market.

Perforated film absorbent dressings	**Absorbent, perforated film with adhesive border**
Melolin (*Smith & Nephew*)	Mepore (*Mölnlycke*)
Release (*Johnson & Johnson*)	Primapore (*Smith & Nephew*)
Skintact (*Robinson*)	
Knitted viscose primary dressings	**Polyurethane foam/film dressing: 1. with adhesive border**
N-A Dressing (*Johnson & Johnson*)	Allevyn Adhesive (*Smith & Nephew*)
N-A Ultra (*Johnson & Johnson*)	Lyofoam Extra Adhesive (*Seton*)
Tricotex (*Smith & Nephew*)	Tielle (Adhesive Margin) (*Johnson & Johnson*)
Vapour permeable adhesive film dressings	**Polyurethane foam/film dressing 2. Without adhesive border**
Bioclusive (*Johnson & Johnson*)	Allevyn (Non-adhesive) (*Smith & Nephew*)
Cutifilm (*Beiersdorf*)	Flexipore (*Polymedica*)
EpiView (*Convatec*)	Lyofoam Extra (Non-adhesive) (Seton)
Mefilm (*Mölnlycke*)	Spyrosorb (Adhesive) (*Perstorp*)
Opsite Flexigrid (*Smith & Nephew*)	
Tegaderm (*3M Health Care*)	
Povidone iodine fabric dressing	**Alginate dressing with absorbent backing**
Inadine (*Johnson & Johnson*)	Sorbsan Plus (Maersk)
Hydrocolloid dressings: 1. Fibrous, No Adhesive Border	**Activated charcoal cloth with silver**
Aquacel (Convatec)	Actisorb Plus (*Johnson & Johnson*)

Table 1. 3. (continuation) Common wound healing products on market.

Hydrocolloid dressings: 2. Semipermeable With Adhesive Border	**Hydrogel dressings**
Comfeel Plus Contour Dressing (*Coloplast*)	AquaForm (Robert Bailey)
Granuflex (Bordered) (*Convatec*)	Granugel Hydrocolloid Gel (*Convatec*)
Hydrocoll Border (*Hartmann*)	Intrasite Gel (*Smith & Nephew*)
Tegasorb Advanced Formulation (*3M Health Care*)	Nu-Gel (Johnson and Johnson)
	Purilon Gel (Coloplast)
	Sterigel (*Seton*)
Hydrocolloid dressings: 3. Semipermeable Without Adhesive Border	**Alginate dressings**
Comfeel (bevelled edge) (*Coloplast*)	Algisite (*Smith & Nephew*)
Comfeel Plus Ulcer Dressing(*Coloplast*)	Algosteril (*Beiersdorf*)
Cutinova Foam (*Beiersdorf*)	Comfeel SeaSorb (*Coloplast*)
Granuflex (Improved Formulation) (*Convatec*)	Kaltogel *(Convatec*)
Hydrocoll Basic(*Hartmann*)	Kaltostat (*Convatec*)
Tegasorb Advanced Formulation (*3M Health Care*)	Melgisorb (*Molnlycke*)
	Sorbsan (*Maersk*)
	Tegagel (*3M Health Care*)
Hydrocolloid dressings: 4. Thin Semipermeable No Adhesive Border	**Hydrocolloid dressings: 5. Thin Semipermeable + Adhesive Border**
DuoDerm Extra Thin (*Convatec*)	Combiderm (Convatec)
Hydrocoll Extra Thin (*Hartmann*)	

1.5. References

1. Enoch, S. and P.E. Price, *Cellular, molecular and biochemical differences in the pathophysiology of healing between acute wounds, chronic wounds, and wounds in the aged.* World Wide Wounds, 2004

2. Badiavas, E.V. and V. Falanga, *Gene therapy.* J Dermatol, 2001. **28**(4): p. 175-92.

3. Eming, S.A., et al., *Genetically modified human keratinocytes overexpressing PDGF-A enhance the performance of a composite skin graft.* Hum Gene Ther, 1998. **9**(4): p. 529-39.

4. Rosenberg, S.A., et al., *Human gene marker/therapy clinical protocols.* Hum Gene Ther, 1999. **10**(18): p. 3067-123.

5. Badiavas, E.V. and V. Falanga, *Treatment of chronic wounds with bone marrow-derived cells.* Arch Dermatol, 2003. **139**(4): p. 510-6.

6. Leigh, I.M., et al., *Clinical practice and biological effects of keratinocyte grafting.* Ann Acad Med Singapore, 1991. **20**(4): p. 549-55.

7. Boyce, S.T., *Design principles for composition and performance of cultured skin substitutes.* Burns, 2001. **27**(5): p. 523-33.

8. Phillips, T.J., *Biologic skin substitutes.* J Dermatol Surg Oncol, 1993. **19**(8): p. 794-800.

9. Mansbridge, J., et al., *Three-dimensional fibroblast culture implant for the treatment of diabetic foot ulcers: metabolic activity and therapeutic range.* Tissue Eng, 1998. **4**(4): p. 403-14.

10. Falanga, V., et al., *Wounding of bioengineered skin: cellular and molecular aspects after injury.* J Invest Dermatol, 2002. **119**(3): p. 653-60.

11. Phillips, T.J., et al., *The longevity of a bilayered skin substitute after application to venous ulcers.* Arch Dermatol, 2002. **138**(8): p. 1079-81.

12. Kearney, J.N., *Clinical evaluation of skin substitutes.* Burns, 2001. **27**(5): p. 545-51.

13. Cullen, B., et al., *Mechanism of action of PROMOGRAN, a protease modulating matrix, for the treatment of diabetic foot ulcers.* Wound Repair Regen, 2002. **10**(1): p. 16-25.

14. Cullen, B., et al., *The role of oxidised regenerated cellulose/collagen in chronic wound repair and its potential mechanism of action.* Int J Biochem Cell Biol, 2002. **34**(12): p. 1544-56.

15. Veves, A., P. Sheehan, and H.T. Pham, *A randomized, controlled trial of Promogran (a collagen/oxidized regenerated cellulose dressing) vs standard treatment in the management of diabetic foot ulcers.* Arch Surg, 2002. **137**(7): p. 822-7.

16. van den Berg, A.J., et al., *A novel formulation of metal ions and citric acid reduces reactive oxygen species in vitro.* J Wound Care, 2003. **12**(10): p. 413-8.

17. Hoang, A.M., et al., *Amelogenin is a cell adhesion protein.* J Dent Res, 2002. **81**(7): p. 497-500.

18. Thomas, S., *Pain and wound management.* Community Outlook, 1989: p. 11-5.

19. Lawrence, J., *The effect of bacteria and their products on the healing of skin wounds, Proceedings of a Symposium.*

. Royal College of Physicians , Andover Medifax, London., 1987.

20. Wilson, P., *Methicillin resistant Staphylococcus aureus and hydrocolloid dressings.* Pharm J, 1988. **241**: p. 787-788.

Chapter 2

Introduction

2.1. Chronic wounds: The health care - an increasing economic problem.

The EU commission (Brussels, December 5, 2001) has stated that the current demographic trends in the EU will lead to a quantitative increase in the demand for health care. As the share of population over 65 is to increase from 16.1% in 2000 to 27.5% in 2050, member countries are to pursue accessibility, quality and sustainability of the health care system. EU Health policy (June, 2004) identified prevention as one of the most effective means to reduce the costs of health care.

Pressure ulcers are a common problem in older patients admitted to hospitals or living in nursing homes. They are a serious problem for the older population since they are responsible for high morbidity, mortality and impaired quality of life [1-3].

Table 2.1. Epidemiological Data on Pressure Ulcers, from [4]

Canada	Prevalence of 26% in all healthcare institutions; 25% in acute care, 30% in long-term sub-acute care and 15% in the community
Germany	Prevalence of 5.3–28.3% in hospitals
Iceland	Prevalence of 8.95%
Italy	Prevalence of 8.3% in hospital settings and over 30% in home-care settings
Japan	Prevalence of 5.1% and incidence of 4.4%
Netherlands	Prevalence of 23.1%
Spain	Prevalence of 9.1% in home care patients, 8.9% in hospitals and 10.9% in residential care
USA	Prevalence in hospital of 15%, incidence of 7%
Europe	Overall prevalence of 18.1% with variations between countries

Chronic wounds represent a major health care problem and impose a significant economic burden. Studies estimate that more than 2 million people are affected by pressure ulcers and another 600,000 to 2.5 million people suffer from leg and foot ulcers of various causes [5]. In agreement with epidemiology indicators, pressure ulcers constitute an important problem with a high variability in their prevalence (see Table 2.1). The annual total cost for treating chronic wounds is in Europe undoubtedly exceeds several thousand million Euros.

Currently, there is not one accepted treatment for this costly medical crisis, which causes a vast amount of amputations and significantly compromises the patient's quality of life.

Complications from pressure ulcers are estimated to result in the deaths of 7% to 8% of persons with spinal cord injury. Thus, good nursing skills, including regular washing and debridement, application of antimicrobials to prevent the infection, and daily changing the dressing, can play a major role in improving the patient's outcome. However, in many cases, the aforementioned procedures are simply not enough to promote wound closure. Intrinsic health factors, such as diabetes, immune status and, in many cases, age are often the major inhibitory factors to wound healing [6]

The magnitude of this problem can be expected to increase as our population ages. This will place a further burden on the health care system. In times of cost pressure, as nowadays, it is very easy to think that money can be saved by reducing staff training, avoiding the need to buy relatively expensive preventative equipment or buying the cheapest products, but when focusing on pressure ulcers it is clear that the consequences of such short-sightedness are higher costs in the long term, inefficient use of healthcare services, frustration in many healthcare professionals, unnecessary pain and discomfort for patients and even avoidable mortality and an increase in litigation against healthcare professionals and institutions [4].

As it is outlined in the state-of-the-art section, wound dressings currently on the market do not fully address all of the physiological issues associated with chronic wounds. Many sophisticated dressings are available for the wound care treatment, made from a wide range of materials including polyurethane, salts of alginic acid and other gelable polysaccharides such as starch and carboxymethylcellulose. These materials may be used alone or in combination to form products as diverse as films, foams, fibrous products, beads, hydrogels or adhesive gel-forming wafers more commonly called hydrocolloid dressings.

Depending upon their structure and composition, such dressings may variously be used to absorb exudates, combat odor and infection, relieve pain, promote autolytic debridement (wound cleansing) or provide and maintain a moist environment at the wound surface to facilitate the production of granulation tissue and the process of epithelization.

Some dressings simply absorb exudates or wound fluid and may therefore be suitable for application to a variety of different wound types. Others have a very clearly defined specialist function and as such have a more limited range of indications. This may mean that they are only suitable for the treatment of specific types of wounds or for the management of a wider range of wounds during a particular phase of the healing cycle.

In 2009 the advanced wound management market was estimated at $5.0 billion, with a grow rate of 4% over the preceding year [7]. It is a huge market and companies are willing to invest large amounts of money into this type of product development. In the state-of-the-art section (table 1.3.), it is showed the main part of released products on the chronic wound management market.

The aging population, along with the increased prevalence of Type II diabetes, has led many to concentrate on improving wound dressing treatments for chronic wounds. The development of innovative multifunctional textiles will lead to radical breakthroughs in prevention and treatment of chronic diseases and thereby realizing important savings and improved quality of life. However, as this is an incredibly complex issue, many factors need to be taken into account. With this in mind, this literature review will provide background information on wound healing; thus will summarize the characteristics of chronic ulcers, including the role of protease activity, myeloperoxidase activity, reactive oxygen species and bacterial infection; and will also focus on functionalization and modification of biopolymeric materials.

2.2. Acute wounds and chronic wounds

From the clinical point of view wounds are generally classified into acute and chronic wounds. Acute wounds, either traumatic or surgical follow the healing process at a predictable rate whereas the non-healing wounds will not progress in a predictable manner but become chronic.

When the skin becomes compromised, the normal wound healing process resolves the injury. Understanding wound healing process will provide knowledge for evaluating the stage at which healing stops, the factors that may have contributed to the disturbing wound healing process and why could end up being a chronic wound.

2.2.1. Normal wound healing

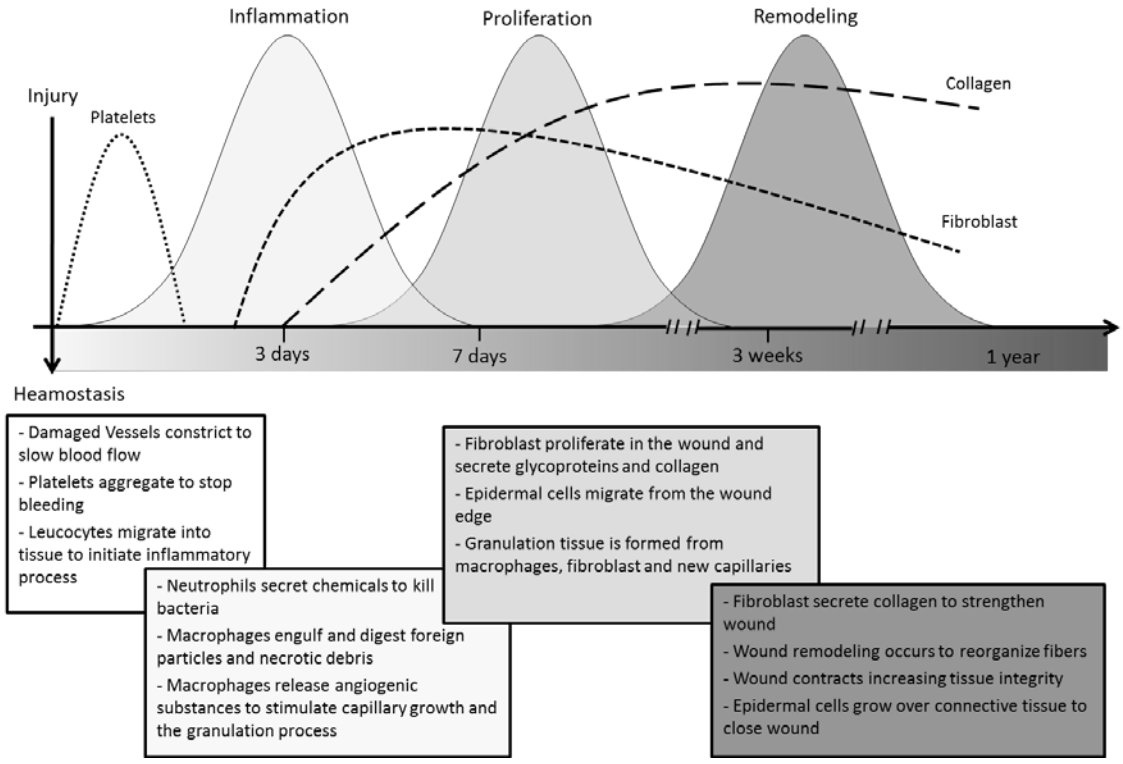


Figure 2. 1. Physiological stages of wound repair and sequence of events during normal wound healing.

The wound healing process is a highly ordered and extremely complex process between orderly expressed cell types, extracellular matrix and an array of soluble mediators, designed to restore

skin integrity. This process involves keratinocyte and fibroblast proliferation, regeneration of the extracellular matrix, connective tissue regeneration, and blood vessel growth.

The classic model of wound healing has been traditionally divided into three or four stages (hemostasis is not considered a phase by some authors): **inflammation**, cellular **proliferation** and tissue **remodeling and maturation.** Although these three stages sequentially follow each other, they are not mutually exclusive and they are overlapped phases by which the body repairs the damaged tissue or organ (see fig 2.1).

When an injury to the skin occurs, the development of inflammatory response by the body immune system is the first step, necessary to debride the wound of foreign material and damaged tissue and to control bioburden. A number of cells of the immune system are involved and these include platelets that form a blood clot, the white blood cells that engulf the microorganisms, as well as the mesenchymal cells that develop into fibroblasts. A reconstructive phase characterized by the deposition of granulation tissue, re-epithelialization and wound contraction, and finally the remodeling phase and scar formation [8].

Recently, a complementary model has been described [9]. In this construct, the process of wound healing is divided into two major phases: early phase and cellular phase:

The early phase, which begins immediately following skin injury, involves cascading molecular and cellular events leading to hemostasis and formation of an early, makeshift extracellular matrix—providing structural support for cellular attachment and subsequent cellular proliferation.

The cellular phase follows the early phase, and involves several types of cells working together to mount an inflammatory response, synthesize granulation tissue, and restore the epithelial layer. Nevertheless, the following text is based on the classic model of wound healing.

2.2.1.1. The inflammatory phase

The inflammatory phase begins immediately after injury can be divided into **bleeding**, **hemostasis** and **inflammation** [10]. It involves predominantly white blood cells such as neutrophils, monocytes, and macrophages. These cells mount an initial defense against microbial invasion and secrete a variety of proteolytic enzymes capable of liquefying nonviable tissue components and microorganisms in the wound area.

The **bleeding** from a fresh wound has the effect of cleansing the wound of some of the invading foreign bodies debris. Within seconds, vasoconstriction, platelet adhesion and aggregation, along with blood coagulation, result the formation of thrombus which encourages hemostasis.

The **hemostasis** begins immediately after wounding, just before the inflammatory phase is initiated, with vascular constriction and fibrin clot formation. The clot provides a scaffold for the migrating cells such as neutrophils, monocytes, fibroblasts, and endothelial cells. The clot and surrounding wound tissue release pro-inflammatory cytokines and growth factors. Once bleeding is controlled, inflammatory cells migrate into the wound (chemotaxis) and promote the **inflammatory phase**, which is characterized by the sequential infiltration of neutrophils, macrophages, and lymphocytes [11-13]. A critical function of neutrophils is the clearance of invading microbes and cellular debris in the wound area, although these cells also release proteases such as Matrix metalloproteinases (MMPs) and serine proteases which can cleave extracellular matrix components in the wound area, and reactive oxygen species (ROS), which cause some additional bystander damage. Thereafter, the tissue and blood monocytes are transformed into macrophages [12]. Macrophages are responsible for inducing and clearing apoptotic cells (including neutrophils), leading to the resolution of inflammation. As macrophages clear these apoptotic cells, they undergo a phenotypic transition to a reparative state that stimulates keratinocytes, fibroblasts, and angiogenesis to promote tissue regeneration [14, 15]. In this way, macrophages promote the transition to the proliferative phase of healing.

2.2.1.2. Proliferative phase

The proliferative phase generally follows and overlaps with the inflammatory phase, and is characterized by **angiogenesis**, **collagen deposition**, **granulation tissue formation** and **re-epithelialization** [16]. In angiogenesis, new blood vessels are formed by vascular endothelial cells [17]. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix (ECM) by excreting collagen, fibronectin as well as glycosaminoglycans and proteoglycans, which are major components of the ECM [16].

While angiogenesis, fibroplasia and granulation tissue formation is taking place in deep tissue, re-epithelialization through migration of epidermal cells helps restore epithelial integrity is taking place on the wound surface, providing cover for the new tissue.

The process of **angiogenesis**, also called neovascularization occurs concurrently with fibroblast proliferation when endothelial cells migrate to the area of the wound, promoting the growth of capillaries into the area of injury. These are essential to bring oxygen and nutrients to the fibroblasts and other cells that are involved in the reconstruction process. Thus angiogenesis is imperative for other stages in wound healing. It is stimulated by macrophages, responding to low oxygen levels in the damaged area. Stem cells of endothelial cells, originating from parts of

uninjured blood vessels, develop pseudopodia and push through the ECM into the wound site to establish new blood vessels [18]. Endothelial cells are attracted to the wound area by fibronectin found on the fibrin scab and chemotactically by angiogenic factors released by other cells [19, 20] e.g. from macrophages and platelets when in a low-oxygen environment. Endothelial growth and proliferation are also directly stimulated by hypoxia, and presence of lactic acid in the wound [21].

To migrate, endothelial cells require the local secretion of proteolytic enzymes, especially MMPs to degrade the clot and part of the ECM [22]. MMPs digest basement membrane and ECM to allow cell migration, proliferation and angiogenesis [22]. When macrophages and other growth factor-producing cells are no longer in a hypoxic, lactic acid-filled environment, they stop producing angiogenic factors [21]. Thus, when tissue is adequately perfused, migration and proliferation of endothelial cells are reduced. When the capillary system is sufficiently repaired and tissue oxygenation and metabolic needs are met, eventually blood vessels that are no longer needed die by apoptosis[23]

Fibroblasts appear shortly after the inflammatory stage, attracted to the area by platelet-derived growth factor, fibroblast growth factor and other mediators. They migrate into the wound area from surrounding tissues and quickly become the predominant cells [24]. After the fibroblasts have migrated into the provisional wound matrix they proliferate and begin to synthesize non-cellular support of ECM structure that includes collagen, elastin, glycoproteins, fibronectin and hyaluronan [20, 25]. This process of fibroblast proliferation and synthetic activity is known as fibroplasia. The new capillaries embedded in a loosely ECM that give granulation tissue its characteristic uneven or granular appearance. – hence called granular tissue.

Granulation tissue consists of new blood vessels, fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and the components of a new, provisional extracellular matrix (ECM). The provisional ECM is different in composition from the ECM in normal tissue and its components originate from fibroblasts. One of fibroblasts' most important duty is the production of collagen. Collagen deposition is important because it increases the strength of the wound. Before it is laid down, the only thing holding the wound closed is the fibrin-fibronectin clot, which does not provide much resistance to traumatic injury. Also, cells involved in inflammation, angiogenesis, and connective tissue construction attach to, grow and differentiate on the collagen matrix laid down by fibroblasts [26].

The formation of granulation tissue in an open wound allows the re-epithelialization phase to take place as epithelial cells migrate across the new tissue to form a barrier between the wound

and the environment. During the **re-epithelialization,** the provisional wound matrix is remodeled and replaced with scar tissue, consisting of new collagen fibers, proteoglycans and elastin fibers, which partially restore the structure and function of the tissue. This is accomplished by the migration, proliferation and differentiation of epithelial cells, dermal fibroblasts and vascular endothelial cells from adjacent uninjured tissue and stem cells that originate in the bone marrow and circulate to the wound site [26].

Fibroblasts in the normal dermis are typically quiescent and sparsely distributed, in contrast to those in the provisional wound matrix and granulation tissue, which are numerous and active. Fibroblasts migrate into the wound in response to soluble cytokines and growth factors, which are initially released from platelets when they degranulate and later by macrophages in the wound [26].

The direction of fibroblast movement is determined by the concentration gradient of chemotactic factors and by the alignment of the fibrils in the ECM and provisional matrix. Fibroblasts tend to migrate along these fibrils, as opposed to across them. They begin moving by binding first to matrix components such as collagen, fibronectin, vitronectin and fibrin via their cell-surface integrin receptors [27]. While one end of the fibroblast remains bound to the matrix component, the cell extends a cytoplasmic projection to find another binding site. When the next site is found, the attachment to the original site is broken by MMPs secreted by the fibroblast, essential for the migration of cells through the ECM. The cell uses its cytoskeletal network of actin fibers to pull itself forward. Partially degraded collagen molecules will not bind (interact) properly with new collagen molecules synthesized during scar formation, resulting in disorganized, weak ECM, so the degraded collagen molecules must be removed by controlled action of the MMPs. This process must be carefully controlled by tissue inhibitors of MMPs (TIMPs) to prevent the MMPs from degrading intact, functional matrix [28].

2.2.1.3. Remodeling phase

Following robust proliferation and ECM synthesis, wound healing enters the final remodeling phase, which can last for years. In the remodeling phase the wound matures. One critical feature of the remodeling phase is ECM remodeling to an architecture that approaches that of the normal tissue. The wound also undergoes physical contraction throughout the entire wound-healing process, which is believed to be mediated by contractile fibroblasts (myofibroblasts) that appear in the wound [11, 13, 29]

Collagen is remodeled and realigned along tension lines where the wound is contracting. During this process the cells that are no longer needed are removed by apoptosis. When the levels of

collagen production and degradation equalize, the maturation phase of tissue repair is said to have begun [21]. During Maturation, type III collagen, which is prevalent during proliferation, is gradually degraded and the stronger type I collagen is laid down in its place [30]. Originally disorganized collagen fibers are rearranged, cross-linked, and aligned along tension lines [21]. Then, the phase progresses, tensile strength of the wound increases. The strength approaches 50% that of normal tissue by three months after injury and ultimately becomes as much as 80% as strong as normal tissue [31]. Since activity at the wound site is reduced, the scar loses its erythematous appearance as blood cells that are no longer needed are removed by apoptosis [21]

2.2.2. Chronic wounds

When the healing process is interrupted, a chronic wound is produced. Chronic wounds have been defined as wounds that have failed to return to functional and anatomical integrity in a timely fashion, or wounds that have proceeded through the repair process without a normal functional end result. All chronic wounds begin as acute wounds and the process of wound healing described above may be hindered and even stopped as a result of various factors.

Such factors include **age** which has a major impact on healing because of decrease in inflammatory response and physiological processes such as blood circulation, reduction in collagen formation, basement membrane degeneration [32]. **Malnutrition** prevents wound healing by decreasing collagen production and other proteins needed for wound repair. **Bacteria** in high concentrations produce toxic end products and compete with cells in the granulation tissue for available nutrients in the wound bed [33]. **Stress** has also been implicated in the impaired healing process. Studies have shown that decreased wound healing is associated with psychological stressors such as pain and noise [34]. Although any wound can become chronic, most chronic wounds are **diabetic foot ulcer**, **venous leg ulcers** and **pressure ulcer**. Although these wounds all have very different etiologies, chronic wound development invariably stems from three factors; the cellular and systemic effects of **aging,** repeated **ischemia–reperfusion injury**, and **bacterial contamination** resulting in an inflammatory response [35]. These factors promote a decreased growth factors, decreased keratinocyte migration, increased reactive oxygen species (ROS), increased tissue proteases, and microbial contamination.

2.3. Pressure ulcers

Pressure ulcer is the most widely used and preferred term for a wound that develops as a result of pressure on a bony prominence that leads to soft tissue damage. Other terms that have been used to describe such wounds are decubitus ulcer, pressure sore, or bedsores. A pressure ulcer is classified as a type of chronic wound due to the time of injury is often unknown, and healing is prolonged and disorganized.

Pressure ulcers occur as result of external forces, pressure, shear, and friction, in combination with patient risk factors that influence tissue tolerance and promote ulcer development. These 3 forces combine to result in an ischemia (inadequate blood supply to an area of skin). Following a localized ischemic injury, a cascade of physiological events occurs characterized by anaerobic metabolism, production of toxic metabolites, acidosis, increased cell membrane permeability, cellular edema, cell death, and finally tissue necrosis. Paradoxically, reperfusion of ischemic tissues introduces an influx of inflammatory mediators and free radicals that can initially promote further tissue damage rather than restore normal function [36, 37]. The cellular injury is known as ischemia-reperfusion injury. This injury promotes the occlusion of blood vessels. Swelling of capillary endothelial cells, plugging of capillaries by leucocytes, fibrin deposits and microthrombi, contributes to the failure of capillary perfusion after prolonged ischemia [38]. Once perfusion decreases, tissue hypoxia occurs, which results in skin necrosis and pressure ulcer formation. Sustained pressure over a bony prominence for 1 to 2 hours can cause tissue damage, and pressure ulcers may develop within 2 to 6 hours. Although injury is often incurred by excessive mechanical loads, studies have indicated that exposure of low pressure over a protracted duration may have the same detrimental effect. Common locations for pressure ulcer formation include the sacrum, trochanter, heels, and elbows [39], as is depicted in figure 2.2.

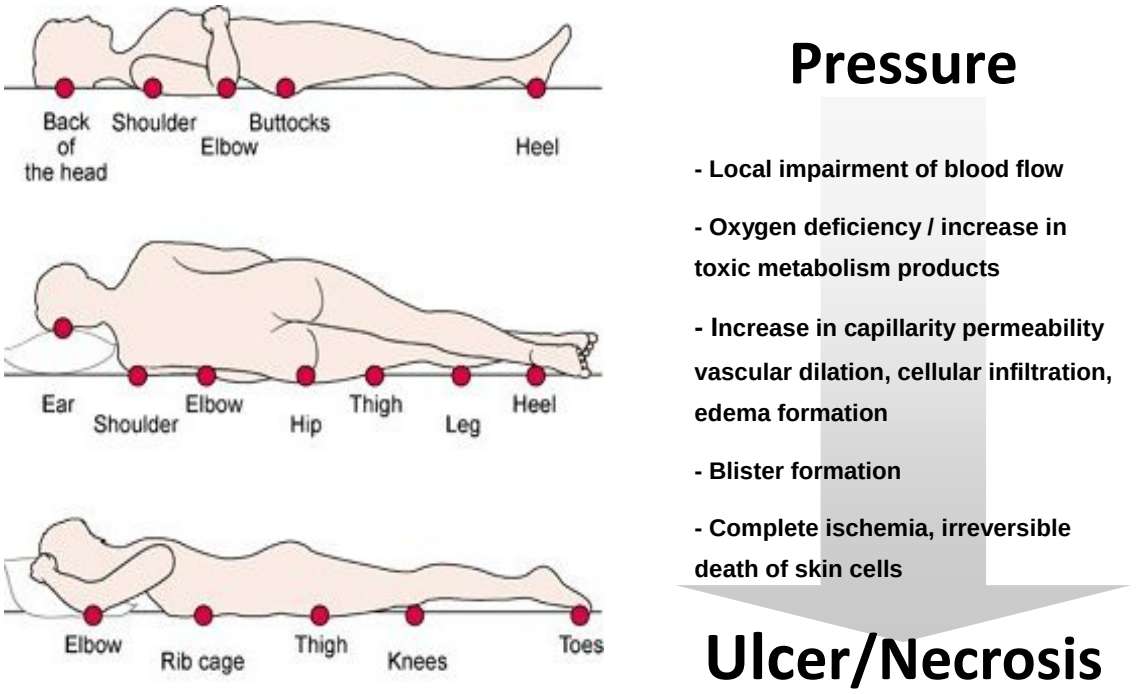


Figure 2.2. Causes of pressure sore: For short periods, the skin can survive exposure even to heavy pressure without being damaged. If the pressure persists, however, the affected skin cells become completely ischemic because of the increasing impairment of blood circulation and the skin cells die. Diagram showing the areas of the body at risk of pressure sores when lying down (from Cancer Help UK, original diagram by the Tissue Viability Society)

2.3.1. Classification and degrees of severity of decubitus

Considering the origins of pressure ulcer, it is clear why the ulceration develops in stages: The longer the area of skin is exposed to pressure, the more severe the tissue damage becomes.

The classification of degrees of severity is therefore based on an evaluation of which layers of skin have already been destroyed by the pressure damage. Various decubitus classifications are used, such as Daniel's classification into five degrees of severity, which is used particularly in the surgical field, or the most commonly used classification into four degrees of severity developed by the "National Pressure Ulcer Advisory Panel" in 1989.

-Stage I: An observable, pressure-related alteration of intact skin, whose indicators as compared to an adjacent or opposite area on the body may include changes in one or more of the following parameters: hyperthermia of the skin (warmth or coolness), induration or edema, tissue consistency (firm or boggy feeling), sensation (pain, itching), and depigmentation.

With consistent pressure relief the reddening pales after several hours or days, depending on the severity of the prior impairment of blood perfusion.

-Stage II: Partial loss of epidermis as far as the dermis. This is a superficial ulcer which may manifest clinically as an abrasion, blister or shallow crater.

-Stage III: Damage to all layers of skin (epidermis, dermis and subcutis), which may extend as far as the fascia beneath the skin, although the fascia is not yet affected. Clinically, the pressure ulcer looks like an open sore with or without undermining of the surrounding tissue.

-Stage IV: Loss of skin involving the entire skin thickness with extensive tissue necrosis and damage to muscles, tendons and bones. Undermining and pocket formation are also commonly seen.

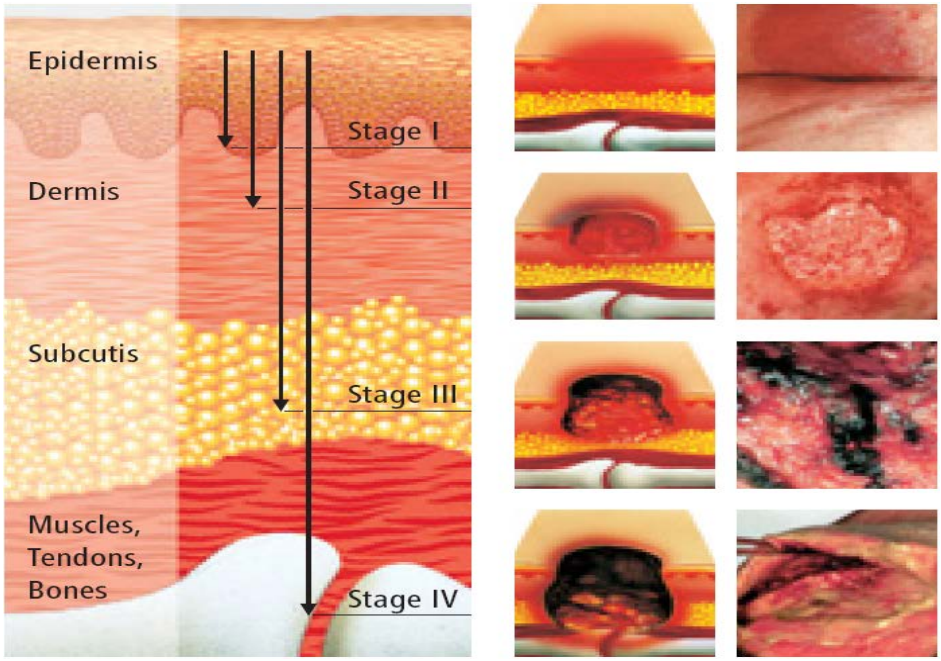


Figure 2.3. Classification of the severity of pressure ulcer is based on which tissue layers have already been destroyed by exposure to pressure. From PAUL HARTMANN AG Phase-specific wound management of decubitus ulcer April 2006 ISBN 3-929870-44-4

2.4. A molecular and biochemical view of chronic wounds /pressure ulcers

2.4.1. Chronic wound environment and impaired healing

Proteolytic degradation of extra-cellular matrix (ECM) proteins is an essential feature of repair and remodeling during cutaneous wound healing. Degradation of ECM by proteinases plays a substantial role in tissue remodeling during normal wound healing. In acute wound a balance between proteinase inhibitors and proteinases activity (e.g. MMPs) regulates the degradation and regeneration of the ECM. Repeated ischemia perfusion events potentiate the cycle of inflammatory cytokines, leukocyte migration, and protease and oxidant injury with loss of tissue perfusion. Thus, after ischemia perfusion injury, these proteolytic events become excessive and are not adequately controlled; this proteolytic imbalance may lead to generalized tissue destruction. Such massive destruction of the ECM is a key factor in the impairment of healing as seen in chronic ulcers [40, 41]. A number of these changes may be further enhanced by environmental factors, such as bacteria and their metabolites [42] and the generation of reactive oxygen species (ROS), within the wound [43, 44]

The over-abundant neutrophil infiltration is responsible for the chronic inflammation characteristic of non-healing pressure ulcers. The neutrophils release significant amounts of oxidative enzymes such as myeloperoxidase (MPO) and proteolytic enzymes such as collagenase (MMP-8) that is responsible for destruction of the connective tissue matrix [45, 46]. In addition, the neutrophils release an enzyme called elastase that is capable of destroying important healing grow factors [47]. Another marker of these chronic ulcers is an environment containing excessive reactive oxygen species (ROS) that further damage the cells and healing tissues [48]. These chronic ulcers will not heal until the chronic inflammation is reduced.

Furthermore, all chronic wounds intrinsically contain bacteria, and the process of wound healing can still occur in their presence. Therefore, it is not the presence of bacteria [49] but their interaction with the host that determines the organisms' influence on chronic wound healing. The accumulation of necrotic tissue or slough in a chronic wound promotes bacterial colonization and bacterial components may contribute to impaired repair mechanisms of the host by interference with cell–matrix interactions or attenuating the inflammatory response [50].

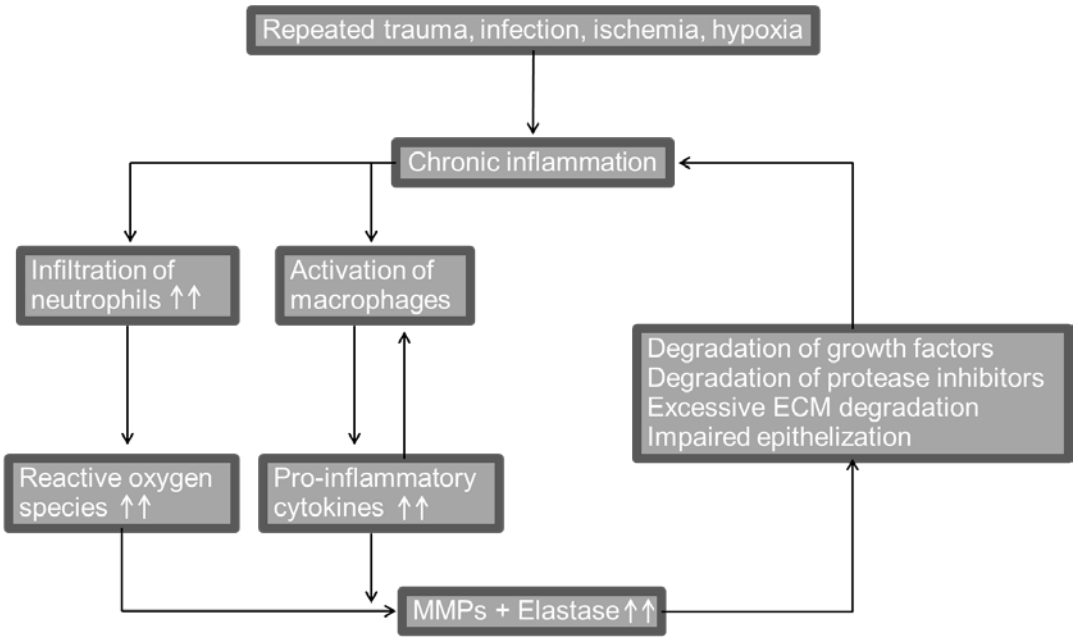


Figure 2.4. Features of chronic wounds. The pathophysiology of chronic wounds is thought to be based on a prolonged inflammatory phase and the destructive processes dominate. Adapted from [51].

2.4.2. The ischemia-reperfusion injury and role of reactive oxygen species

The ischemia-reperfusion injury leads to a decrease in oxygen tension in the cells results in the activation of control mechanisms to maintain steady state. With the restoration of the blood supply and reintroduction of oxygen, toxic oxygen derived free radicals may be formed. Under normal circumstances oxygen-derived free radicals are buffered by free-radical scavenging mechanisms. However, when there is an overproduction of reactive oxygen species (ROS), or a decrease of scavengers as in oxidative stress, free radicals can initiate a sequence of biochemical and cellular events, causing extensive damage to the surrounding tissues [52]. The failure to reperfuse, the so-called, no-reflow phenomenon, is also a result of overproduction of free radicals [53, 54]. An important source of free radicals is derived from the neutrophilic leucocytes. While they fight pathogens, neutrophils also release inflammatory cytokines and enzymes that damage cells [35, 55]. One of their important jobs is to produce ROS to kill bacteria, for which they use an enzyme called myeloperoxidase (MPO) [35]. The enzymes and ROS produced by neutrophils and other leukocytes damage cells and prevent cell proliferation and wound closure by damaging DNA, lipids, proteins[56], the extracellular matrix (ECM), and cytokines impairing the wound healing [35].

2.4.3. Alterations in protease activity, the role of MMP's

Alteration of the ECM is integral to the resolution of wound healing but also has implications in regulation of inflammation. MMPs are key regulators of multiple aspects of tissue repair and the study of these enzymes will provide insight into possible therapies.

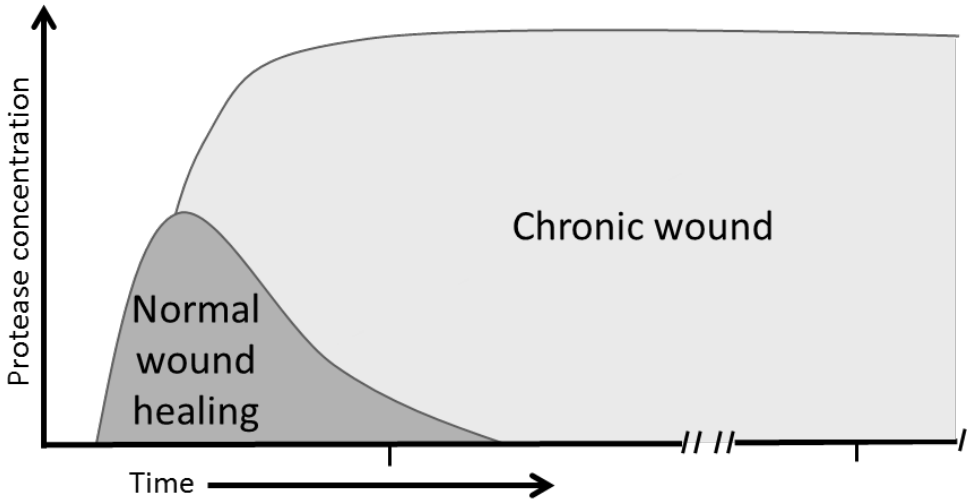


Figure 2.5. Protease levels in normal and chronic wounds

1.4.3.1. MMPs and their role in the normal wound healing process

The MMPs are zinc-dependent endopeptidases collectively capable of degrading essentially all components of ECM [57]. Controlled breakdown of ECM by MMPs plays an important role in detachment and migration of cells, as well as in tissue remodeling in several physiological situations, e.g. developmental tissue morphogenesis, tissue repair, and angiogenesis. It is likely that in these situations distinct MMPs cooperate in parallel and/or cascade- like fashion to achieve effective and targeted ECM degradation, which is also controlled by specific tissue inhibitors of metalloproteinases (TIMPs). In skin, several different types of cells are capable of producing MMPs: keratinocytes, fibroblasts, macrophages, endothelial cells, mast cells, eosinophils, and neutrophils.

MMPs play essential and beneficial roles in normal wound healing processes. MMP enzyme family may theoretically be involved in various tasks during wound repair summarized in table 2.2.

Table 2.2. Main roles of MMPs in normal wound healing. Adapted from [58]

Main phase of healing	Role of MMPs
Inflammation	Removal of damaged ECM (aids autolytic debridement)
Proliferation	Degradation of capillary basement membrane for angiogenesis Aiding detachment and migration of cells
Remodeling	Contraction of scar ECM Remodeling of scar ECM
Wound healing	Regulation of activities of certain growth factors [59]

Removal of damaged ECM [58]

MMPs break down the damaged ECM that occurs at the edge of acute skin wounds. This enables new ECM components (e.g. collagen, fibronectin, and proteoglycans) synthesized by wound cells to integrate correctly with intact ECM components at the wound edges.

In addition, MMPs help to slough out bacterial biofilms. The MMPs secreted by inflammatory cells surrounding biofilms digest (loosen) the attachments between the bacterial biofilms and the wound bed.

Angiogenesis

MMPs degrade the basement membrane that surrounds capillaries. This allows vascular endothelial cells to migrate from capillaries near the wound and to establish new blood vessels into the wound bed [60, 61]

Migration of cells

MMPs (especially MMP-1) are required for migration of epithelial cells, fibroblasts, and vascular endothelial cells across or through the ECM. When epithelial cells at the edge of a wound begin to proliferate and migrate as a sheet across the wound bed, the epithelial cells just trailing behind the leading edge of the sheet secrete MMP-1. This partially digests the type 1 collagen and weakens the attachment of the cells' membranes to the matrix, allowing the cells to move across the collagen matrix [61, 62]

Contraction

MMPs secreted by myofibroblasts are necessary for contraction of newly synthesized scar ECM. Large excision wounds in humans can contract up to about 20% of the initial wound area [63, 64]

Scar remodeling

Repair of skin wounds initially produces a highly disorganized scar matrix. However, wound cells continue to produce low levels of MMPs long after the initial scar is formed. These MMPs slowly remove the disorganized ECM, which is gradually replaced by ECM with a more normal and more highly organized structure [65, 66]

2.4.3.2. MMPs in chronic wound

However, when the level of protease activity is too high the delicate balance between tissue breakdown and repair is disturbed. This unbalanced proteolytic activity, mainly due to excessive MMPs, is the key factor responsible of the persistent inflammatory response at the wound site, which impairs local tissue protective mechanisms leading to a non-healing wound. [67-69] It is currently accepted that in addition to their involvement in protein breakdown of the extracellular network, excessive metalloproteinases also break down growth factors [41, 47].

After reperfusion, in response to pro-inflammatory cytokines, the activated leukocytes (neutrophils and macrophages) adhere to the endothelial cell, release protease enzymes causing inflammation with significant cellular and tissue injury [70].

Pro-inflammatory cytokines are considered as potent inducers of MMP expression in chronic wounds, and they have been shown to down regulate the expression of tissue inhibitor of metalloproteinases (TIMPs), thus creating an environment with a relative excess of MMP activity. Pressure ulcers are characterized by significantly higher levels of MMP-1 and MMP-8 and lower levels of TIMP-1 than normal wounds. Interestingly, in normal healing wounds, collagenases are present almost exclusively in their inactive forms, whereas nonhealing ulcers possess significant levels of the active enzymes [40, 46]. In addition, elevated levels of various serine proteinases have been found at the chronic wound site, particularly of neutrophil origin. These include particularly neutrophil elastase [25, 69]

Indeed, the major protease inhibitors, as well as components of the provisional wound matrix, have been shown to be degraded and inactivated within the chronic wound environment [25]. Growth factors pivotal for repair are targets of wound proteases, and they are inactivated by proteolytic cleavage [23, 71]. In summary, the involvement of excessive MMPs in wounds contributes to the development of delayed healing, via three mechanisms:

- Protein breakdown of components of the neo-matrix,
- Breakdown of growth factors,

- Breakdown of TIMP proteins, thereby blocking any feedback mechanism linked to proteinase hyperactivity.

Consequently, one of the current treatment strategies in the management of chronic wounds is directly aimed at restoring the proteolytic unbalance of MMPs. However, since MMP activity is necessary for normal wound healing, to ascertain the potential of MMP inhibitors as therapeutics, it is crucial to understand MMPs mode of action.

2.4.3.3. MMP, chemical structure and mode of action

MMPs have since been identified as the major enzymes responsible for turnover of extracellular matrix by proteolytic degradation of virtually all proteinaceous components of the ECM [57]. At present, the MMPs consist of 23 distinct proteases in humans (see table 2.3.), which can be divided into subgroups of collagenases, gelatinases, stromelysins, and membrane type MMPs (MT-MMPs) according to their substrate specificity and primary structure (see figure 2.6.).

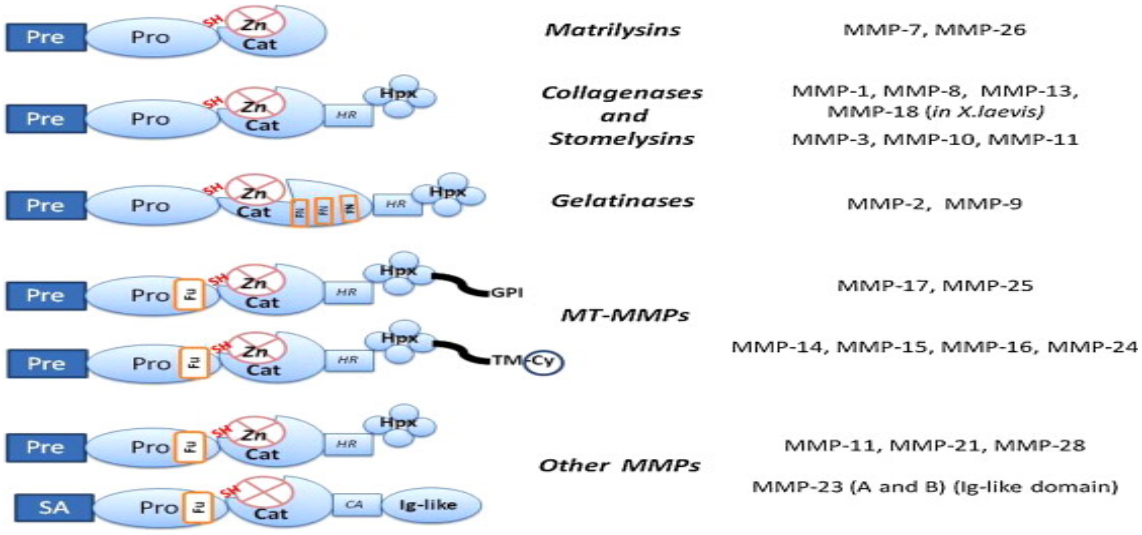


Figure 2.6. Domain structure of secreted and membrane-bound Matrix metalloproteinases. MMPs are comprised of different subdomains and have the "minimal domain" in common, which contains three principal regions: an aminoterminal signal sequence (Pre) to be cleaved by the signal peptidase during entry into the endoplasmic reticulum, a pro-domain (Pro) containing a thiol-group (-SH) and a furin cleavage site, and the catalytic domain with a zinc-binding site (Zn^{2+}) [72].

Table 2.3. Overview of the identified MMPs and their common names. From [73]

MMP	Alternative name	MMP	Alternative name
1	Collagenase-1 Interstitial collagenase	16	Membrane type-3 MMP
2	Gelatinase A 72-kDa type IV collagenase	17	Membrane type-4 MMP
3	Stromelysin-1 Transin-1	18	Human ortholog of *Xenopus* MMP-18
7	Matrilysin Pump-1	19	RASI-1, occasionally referred to as stromelysin-4
8	Collagenase-2 Neutrophil collagenase	20	Enamelysin
9	Gelatinase B 92-kDa type IV collagenase	21	X-MMP Human ortholog of *Xenopus* xMMP
10	Stromelysin-2	23	Cysteine array MMP Femalysin MMP-22
11	Stromelysin-3	24	Membrane type-5 MMP
12	Macrophage metallo-elastase	25	Membrane type-6 MMP Leukolysin
13	Collagenase-3	26	Matrilysin-2/endometase
14	Membrane type-1 MMP	27	C-MMP
15	Membrane type-2 MMP	28	Epilysin

The majority of zinc-dependent metallopeptidases (with the notable exception of the carboxypeptidases) share a common pattern of primary structure in the part of their sequence involved in the binding of zinc, and can be grouped together as a superfamily, known as the metzincins, on the basis of this sequence similarity. Besides their differential domain structure, MMPs can be principally divided into secreted and membrane anchored proteinases.

The MMP catalytic site contains a zinc ion coordinated by three imidazole nitrogens, a glutamic acid side chain suitably oriented to serve as an acid-base catalyst, and a main chain carbonyl that acts as a critical hydrogen bond acceptor.

The most important of these MMPs are collagenase (MMP-1), which cuts intact collagen at a single site; gelatinases (MMP-2 and MMP-9), which degrade partially denatured collagen (gelatin); and stromelysin (MMP-3), which degrades multiple protein substrates in the ECM.

2.4.3.4. The cysteine-switch

ProMMPs are kept in a catalytically inactive state by the interaction between the thiol of the conserved prodomain cysteine and the zinc ion of the catalytic site. For a proMMP to become catalytically active, the thiol–Zn^{2+} interaction must be disrupted. Van Wart and Birkedal-Hansen [74] proposed this process as a general and required step in the activation of all proMMPs, and they termed this mechanism the "cysteine-switch", a name that remains valid and widely accepted. In essence, the thiol–Zn^{2+} interaction can be broken – and a latent MMP can gain catalytic activity – by three mechanisms (Figure 2.7).:

- Modification of the free thiol by physiological (oxidants, disulfides, electrophiles) or non-physiologic compounds (alkylating agents, heavy metal ions)[75];
- Direct cleavage of the prodomain by another proteinase, or
- Chemical or allosteric perturbation of zymogen leading to inter or intramolecular autolytic cleavage of the prodomain.

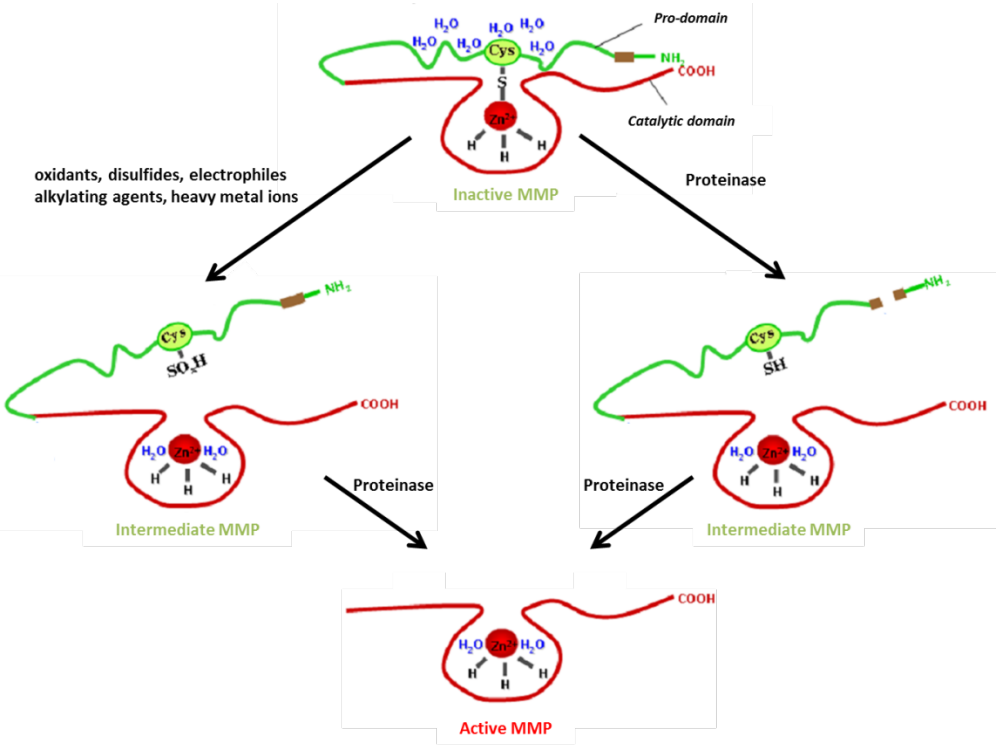


Figure 2.7. Activation of MMPs via the 'cysteine-switch' mechanism. The active site of MMPs consists of 3 histidine (H) residues ligated to the catalytic Zn^{2+} (3His-Zn^{2+}) or Ca^{2+} ions (not shown) within the catalytic domain. Potential substrates are unable to access the active site as it is blocked by the pro-domain and the thiol-Zn^{2+} interaction (i.e. Cys-S-Zn^{2+}). MMPs are activated by modifying or interrupting the thiol-Zn^{2+}interaction either chemically by ROS, disulfides, electrophiles, alkylating agents, heavy metal ions or physiologically by proteinases (such as elastase, plasmin, uPA, furin and other active MMPs) produces an intermediate MMP. Consequently the cleavage of the N-terminal pro-domain by proteinases or by autolysis (e.g. via MMP intermediate itself) produces an active MMP. Figure adapted from [76].

2.4.3.5 MMP's and MPO

Myeloperoxidase (MPO), a heme enzyme released by activated neutrophils, from intracellular granules. This enzyme is the most abundant protein in neutrophils, accounting for up to 5% of their dry mass [77]. It is also present in monocytes, though at lower levels and it is an important source of reactive oxygen species [78]. Levels of myeloperoxidase are significantly higher in the tissues of pressure ulcers compared with those of acute healing wounds [79].

MPO protein has little bactericidal effect per se, but enzymatic reaction with H_2O_2 and halide (Cl^-, Br^-, I^-) ions generates hypohalous acids as hypochlorous acid (HOCl), [80]. This oxidant is widely believed to be responsible for much of the anti-bactericidal activity of neutrophils. However, evidence has emerged that MPO-derived oxidants are extremely cytotoxic and react readily with most biological molecules contributing to tissue damage and the initiation and propagation of chronic inflammatory disease. Myeloperoxidase catalytic activity is a complex phenomenon involving two distinct pathways (Figure 2.8.).

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$$

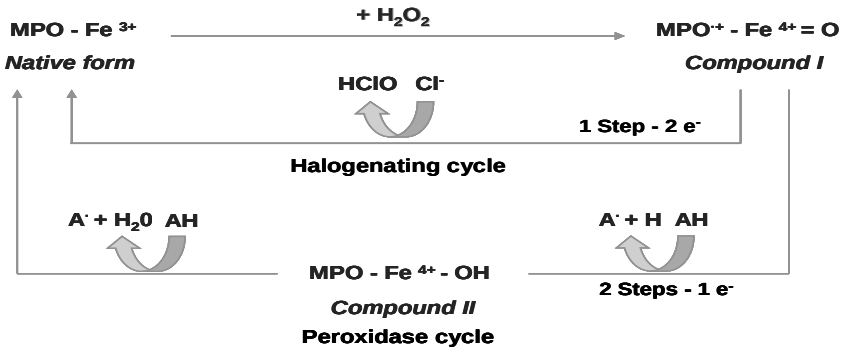


Figure 2.8. Representation of MPO redox transformations and catalytic pathways

The levels of MMPs and characteristic amino acid oxidation products of myeloperoxidase are greatly elevated in inflammatory tissue of humans [81-84]. These observations suggest that HOCl production by myeloperoxidase may provide a pericellular mechanism for activation of latent MMPs during inflammation.

HOCl reacts rapidly and nearly quantitatively interacts with thiols, thioethers, and amino groups [85, 86]. The high susceptibility of Cys residues to oxidation by HClO has important biological implications as this can disrupt the cellular redox balance of cells and inactivate multiple cellular enzymes that contain active site Cys residues. In pressure ulcers, HOCl can activate latent MMPs (e.g., MMP-7) via conversion of a key Cys residue in the "cysteine switch"

domain of pro-MMP-7 to a sulfinic acid [87]. In turn, modification of the prodomain thiol would disrupt the native conformation of the prodomain, allowing autolytic cleavage and activation. These findings suggest that HOCl production by MPO might be a physiologically relevant pathway for triggering MMP activation.

In addition HOCl has important effect on the specific endogenous tissue inhibitors of MMPs (TIMPs). TIMPs inhibit the proteolytic activity of MMPs by forming a stable, noncovalent 1:1 stoichiometric complex [88-90]. Importantly, all TIMPs have a conserved N-terminal sequence that contains Cys [88]. The N-terminal α-amino and carbonyl groups of the Cys residue coordinate the essential catalytic Zn^{2+} of the metalloproteinase indicating that Cys plays a key role in inhibiting MMP activity. Studies have demonstrated that the loss of TIMP inhibitory activity is strongly correlated with Cys oxidation by HOCl generated by MPO [91].

In summary, the involvement of excessive production of HClO by MPO pressure ulcers contributes to the development of proteolytic unbalance in the wound, via two mechanisms:

- Activating the inactive pro-forms of MMPs,
- Impairing the ability of TIMP to regulate MMPs activity

2.4.3.6. The microbiological environment

There are several factors known to affect the bacterial burden of chronic wounds and increase the risk of infection. These include the number of microorganisms present in the wound, their virulence and host factors. The presence of microorganisms alone (colonization) does not indicate an infection in pressure ulcers. Colonization with bacteria is common and unavoidable. All chronic wounds become colonized, usually with skin organisms, followed in 48 hours by gram-negative bacteria.

Wound infection is defined as the presence of replicating microorganisms within a wound with a subsequent host response that leads to a delay in healing. Because of this it is important that infection is recognized as early as possible. The signs and symptoms of local infection are redness (erythema), warmth, swelling, pain, and loss of function. Foul odor and pus may accompany this. Eventually, the local bacterial burden will increase further and becomes systemically disseminated resulting in sepsis, which if not actively treated could progress to multi-organ failure and death in some instances.

The mix of microorganisms within a wound may also be an issue. Most chronic wounds are polymicrobial, and infections generally involve mixed populations of aerobic and anaerobic bacteria. [92, 93]. This is significant because some combinations of bacterial species may

develop synergy with each other, resulting in previously non virulent organisms becoming virulent and causing damage to the host [94-97].

The characteristics of bacteria in chronic wounds are summarized in table 2.4.

Table 2.4. Feautures of bacterial environment in chronic wounds.

High levels of bacterial content (>10^5 bacteria per gram tissue can cause clinical infection)
Presence of more than one bacterial strain
Bacteria capable of altering their phenotypic and genotypic characteristics
Presence of multi-drug resistant organisms
Presence of 'biofilms'
Recurrent local and/or invasive wound infections

Besides to episodes of infection, the continued presence of bacteria in a wound leads to endotoxin production and stimulates the host's immune defenses to produce pro-inflammatory mediators. Although inflammation is part of normal wound healing, the repair process may be prolonged if the inflammation is excessive [98]. Furthermore, with chronic colonization bacteria in wounds form biofilms (bacterial colonies embedded in a self-secreted extracellular polysaccharide matrix) that are resistant to the action of host defenses and antimicrobial agents, thereby contributing to delayed healing [99]. The presence of bacteria induces the immigration of monocytes, macrophages and leukocytes, whose inflammatory response exaggerates the tissue damaging processes [100, 101].

Among all bacterial species found in infected chronic wound, recent findings pointed out to *Pseudomonas aeruginosa* and S*taphylococcus aureus* as main strains involved in and contribute to the lack of healing [102].

S. aureus is most commonly isolated from chronic wounds [103-106] and can be found in superficial zones of chronic wounds and, in certain situations, can express a number of potential virulence factors and surface proteins which promote its adherence to the damaged tissue and decrease neutrophil functions and immune responses of the host [107, 108].

P. aeruginosa, located in the deeper regions of chronic wounds may play an important role to delay or even prevent the healing process [105, 106, 109]. *P. aeruginosa* often causes biofilm-based chronic infections and expresses virulence factors, in particular, rhamnolipid, that can eliminate the activity of PMNs [110, 111]. A number of studies have demonstrated that *P. aeruginosa* is frequently present in chronic wounds [105, 111] and have provided evidence that

the bacteria are located in aggregates enclosed in extracellular polymeric matrix material as found in biofilms [111]. Furthermore, chronic wounds that harbored *P. aeruginosa* were larger than those that did not, and the healing process also seemed to be more severely hindered for those wounds [105, 106, 109].

On the other side, in addition to human MMPs, bacterial proteases have been found to be influential in tissue breakdown and, as such, have a role to play in the healing of infected wounds. Several studies have suggested that bacterial-derived secreted proteases may act to up-regulate the levels of MMPs produced by the host cells [112, 113]. These findings indicate that bacterial protease production should be taken into consideration. Thus, both host MMPs and those derived from infecting bacteria need to be targeted in order to increase the healing capacity of the injured tissue [113].

2.5. Adopted strategies. Selection of the biopolymer platform

In summary, chronic wound environment is characterized with bacterial infection and continuing influx of polymorphonuclear neutrophils that release high concentrations of matrix metalloproteases (MMPs), myeloperoxidase (MPO) and reactive oxidative species causing excessive degradation of the extracellular matrix (ECM) and the growth factors. In healing wounds the MMPs are counteracted by their natural inhibitors, while in chronic wounds the ratio proteases/inhibitors is disturbed and most of these enzymes are uninhibited. The protease-antiprotease unbalance is further promoted by MPO-generated hypochlorous acid (HOCl), which from one side inactivates the protease inhibitors and from another triggers the activity of latent MMP. In addition, most chronic wounds are colonized with several bacterial species, predominantly S*taphylococcus aureus* and *Pseudomonas aeruginosa* that may play an important role to delay or even prevent the healing process.

The development of interactive devices, that promote healing by correcting imbalances of the wound environment, may represent a most appropriate approach to wound healing. Hence, in order to improve the opportunity for wound healing, it is necessary to create conditions that are unfavorable to micro-organisms and favorable for the host repair mechanisms. Furthermore, the modulation of the proteolytic unbalance in the wound and the scavenging of ROS, are also key points to stop the ongoing inflammatory processes, facilitate re-epithelization, and thus support the healing process.

Table 2.5. Performance requirements of the ideal dressing [114]

Primary requirements
Free of toxic or irritant extractables
Does not release particles or non-biodegradable fibres into the wound
Forms an effective bacterial barrier (effectively contains exudates or cellular debris to prevent the transmission of microorganisms into or out of the wound)
If self-adhesive, forms an effective water-resistant seal to the periwound skin, but is easily removed without causing trauma or skin stripping
Maintains the wound and the surrounding skin in an optimum state of hydration (this implies it is able to function effectively under compression)
Requires minimal disturbance or replacement
Protects the periwound skin from potentially irritant wound exudates and excess moisture
Produces minimal pain during application or removal as a result of adherence to the wound surface
Maintains the wound at the optimum temperature and pH

Table 2.5 (continuation) Performance requirements of the ideal dressing [114]

Secondary requirements
Possesses antimicrobial activity — capable of combating localised infection
Has odour-absorbing/combating properties
Able to remove or inactivate proteolytic enzymes in chronic wound fluid
Haemostatic properties
Exhibits effective wound cleansing (debriding) activity

There is a large list of performance requirements of the ideal dressing summarized in table 2.5. Taking into consideration the biochemical chronic wound environment, and keeping these "ideal performance properties" in mind, this Thesis is focused in the development of chronic wound dressings targeting the following key properties:

Ability to adsorb and neutralize free radicals

Ability to balance the proteinase activity by:

- Balance the MMP's activity, cause of the proteolytic environment responsible of the ECM degradation.
- Balance the MPO activity, responsible of:
  - Activating the inactive pro-forms of MMPs,
  - Impairing the ability of TIMP to regulate MMPs activity
  - Oxidative environment.
- Ability to inhibit bacterial growth, spatially S*taphylococcus aureus* and *Pseudomonas aeruginosa*.

To meet all these necessary requirements in a successful approach, the design of the matrices should be based on materials that possess the most part of required properties and capable to be further modified and/or implemented with active molecules to improve its healing properties.

Natural polymer-based systems constitute an interesting alternative to produce novel wound dressing materials. These systems offer the advantage of being similar to biological macromolecules, mimicking the tissue microenvironment and stimulate appropriate physiological responses required for wound regeneration. Owing to their similarity with the extracellular matrix (ECM), natural polymers may also avoid the stimulation of chronic

inflammation or immunological reactions and toxicity, often detected with synthetic polymers. Thus the ideal biomaterial should be non-toxic, biocompatible, promoting favorable cellular interactions and tissue development, while possessing adequate mechanical and physical properties. In addition, it should be biodegradable and bioresorbable to support the reconstruction of a new tissue without inflammation [115, 116]. The resulting materials can be conjugated as membranes, hydrogels, scaffolds and microspheres to be used in specific biomedical applications

Moreover the available biomaterial should require minimal processing, possess antimicrobial properties and promote wound healing. In recent years, a large number of research groups are dedicated to produce a new, improved wound dressing by synthesizing and modifying biocompatible materials [117]. Recent reports are also aiming on the acceleration of the wound repair by systematically designed dressing materials. In particular, efforts are focused on the use of biologically derived materials such as, chitin and its derivatives, which are capable of accelerating the healing processes at molecular, cellular, and systemic levels. Chitin and its derivative, chitosan, are biocompatible, biodegradable, nontoxic, anti-microbial and hydrating agents. Due to these properties, they show good biocompatibility and positive effects on wound healing.

2.5.1 Chitosan, the biopolymer platform

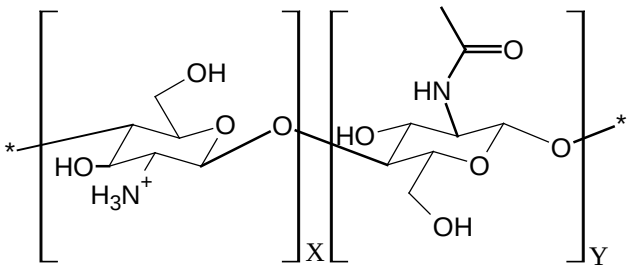


Figure 2.9. Molecular structure of chitosan.

Chitin [poly-β-(1→4)-N-acetyl-D-glucosamine] is an ubiquitous biopolymer found in the exoskeleton of insects, marine invertebrates and fungi and is the second most abundant biomaterial. Chitosan and is prepared by de-N-acetylation of chitin. Chitosan is also naturally present in some microorganisms and fungi.

Chitosan is a linear is a poly-β-(1→4)-D-glucosamine (polysaccharide similar to cellulose) that is obtained by the partial deacetylation of chitin.

Because chitin deacetylation is incomplete, chitosan is formally a copolymer composed of glucosamine and N-acetylglucosamine. It is important to note that the term "chitosan" does not refer to a single well defined structure, and chitosans can differ in molecular weight, degree of acetylation, and sequence (i.e., whether the acetylated residues are distributed along the backbone in a random or blocky manner). As a result of these structural differences, the properties of chitosan (e.g., the pKa) can also vary somewhat.

The unique structural feature of chitosan is the presence of the primary amine at the C-2 position of the glucosamine residues. Few biological polymers have such a high content of primary amines, and these amines (acetyl-D-glucosamine residues), lead to differing physicochemical properties and biological responses to chitosan.

Although chitosan is insoluble in neutral or alkaline aqueous solution, it dissolves in dilute acid solutions, such as hydrochloric, lactic, and acetic acid.

Due to its natural abundance and specific biological properties, chitosan is an attractive material for large spectra of biomedical applications including wound healing.

2.5.2. Chitosan, properties for wound healing applications and regenerative medicine

Compared to common polysaccharides, chitosan and chitin-based products provide improved healing of wounds. These healing effects can be attributed to the biochemical properties of chitosan and its derivatives.

Biocompatibility

Chitosan is reported to be biocompatible [118-123], biodegradable [121, 124-126] and of low toxicity [127]. Chitosan is degraded by lysozyme, and the degradation is slower, both *in vitro* and *in vivo*, for more highly deacetylation samples [119, 126, 128, 129]. The cytotoxicity of chitosan is reported to be dose-dependent and decreases with a decrease in molecular weights and degrees of de-N-acetylation [130]. Additionally, chitosan has partial structural similarities

to glycosaminoglycans, which are essential structural elements of the extracellular matrix of most tissues.

Antibacterial activity

Chitosan inhibits the growth of a wide variety of bacteria [131] (Table 2.6.). In contrast to other type of antibacterial products, chitosan has several advantages because it possesses a higher antibacterial activity, a broader spectrum of activity, a higher killing rate, and a lower toxicity toward mammalian cells [132].

Table 2.6. The antibacterial activity of chitosan. Modified from [132]

Gram-positive bacteria	**(CS%, w/v)**	**Gram-negative bacteria**	**(CS%, w/v)**
Staphylococcus aureus	0.05	*Escherichia coli*	0.025
Corynebacterium	0.025	*Pseudomonas aeruginosa*	0.0125
Staphylococcus epidermidis	0.025	*Proteus mirabilis*	0.025
Enterococcus faecalis	0.05	*Salmonella enteritidis*	0.05
Candida albicans	0.1	*Enterobacter aerogenes*	0.05
Candida parapsilosis	0.1		

Many intrinsic and extrinsic factors can influence the antimicrobial properties, such as the molecular weight, degree of de-N-acetylation, microorganism species, presence or absence of metal cations, etc…. Also, the physical state of the chitosan can present very different antimicrobial properties, such as whether the chitosan is present in the form of films, hydrogels, coatings, in solutions or in combinations with other materials.

Several mechanisms have been proposed for the antimicrobial activity by chitosan but the exact mode of action is still uncertain.

Inhibition of MMPs [133]

Chitosan is reported to inhibit the activation and expression of MMP2 in primary human dermal fibroblasts; therefore chitosan may prevent and treat several health problems mediated by MMP2 (that can hydrolyze the basement membrane collagen IV) such as wound healing. It was speculated that the inhibitory effect might be explained by the effective chelating capacity of chitosan for Zn2+ that would become unable to exert correctly its cofactor role in MMP2 [134, 135]. In addition the partially hydrolyzed chitosans were found to be potent inhibitors of gene and protein expression of MMP9 [136, 137] that increases in the majority of malignant tumors and plays a major role in the establishment of metastases.

It is also found, that glucosamine, a constituent monomer of chitosan, inhibited the expression and the synthesis of MMP3 [138].

Wound healing properties

Chitosan possess the characteristics favorable for promoting rapid dermal regeneration and accelerated wound healing. It is observed that chitosan oligosaccharides have a stimulatory effect on macrophages, and is chemo attractant for neutrophils *in vitro* and *in vivo*, an early event essential in accelerated wound healing. It stimulates cell proliferation and histoarchitectural tissue organization. Chitosan is a hemostat, which helps in natural blood clotting and blocks nerve endings and hence reducing pain [139, 140]. Chitosan can activate biological defense mechanisms. For example, chitosan (ca. 70% de-N-acetylated) showed macrophage activation and cytokine production, to initiate the healing process [141]. Chitosan will gradually depolymerize to release N-acetyl- β-D-glucosamine, which initiates fibroblast proliferation and helps in ordered collagen deposition and stimulates increased level of natural hyaluronic acid synthesis at the wound site [142]. At the final stage of wound healing process, chitosan has been demonstrated to stimulate angiogenesis, reorganization of the extracellular matrix and stimulating granulation tissue formation and re-epithelization [143, 144].

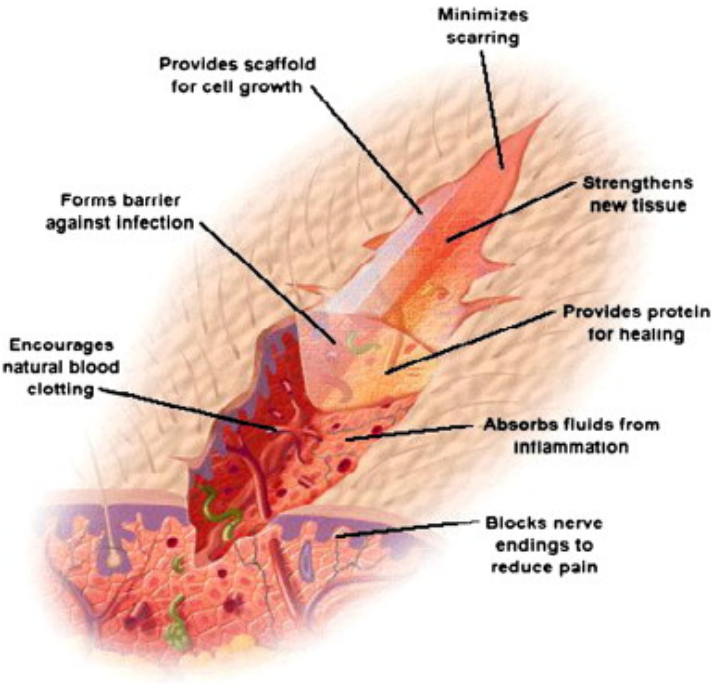


Figure 2.10. Schematic representation of the required properties of wound dressing material (Paul and Sharma, 2004).

Scaffold for the Regeneration of Tissue

Chitosan provides a non-protein matrix for 3D cell proliferation and tissue growth. The advantage of chitosan is that it easily can be processed into multiple scaffolds (summarized in [140]) such as hydrogels, membranes, nanofibers, beads, micro/nanoparticles, micropore scaffolds and sponges. These scaffolds can be modified to yield polymer blends, which are formed with desired structures, chemical features, and mechanical properties for various types of biomedical applications such as wound healing, tissue engineering and drug delivery.

Chitosan chemistry

Chitosan has a versatile chemistry due to the presence of free amino groups in the C2 positions. The active primary amino groups on the molecule being reactive provide sites for a variety of side group attachment employing mild reaction conditions. [145]. The modifications on chitosan provide versatile materials with specific functionality, and tunable biological and physical properties.

2.6. Summary

With so many products coming onto the market, the difficulty is in choosing the optimum treatment for the requirements of each patient. As no one dressing is suitable for the management of all types of wounds, and few are ideally suited for the treatment of a single wound during all stages of the healing cycle, successful wound management depends upon a flexible approach to the selection and use of products based upon an understanding of the healing process combined with a knowledge of the properties of the various dressings available. Without such knowledge and careful consideration of all the factors described above, dressing selection is likely to be arbitrary and potentially ineffective, wasteful both in terms of time and physical resources. Hence, the development of new wound dressings should continue looking for more versatile dressings.

Thus, the new trends in wound care research is developing advanced wound care products to control the biochemical environment of elevated levels of MMPs, MPO, ROS and bacterial load found in chronic wounds. This is because all this factors are found to be detrimental to ECM proteins or factors causing a prolonged inflammatory/ proliferation phases resulting in the development of chronic wounds.

Functionalizing chitosan platforms with active agents enables the generation of highly exclusive/specific bioresponsive wound dressings to modulate the biochemical wound bed microenvironment to facilitate and stimulate wound healing.

This thesis proposes different synthetic routes to functionalize chitosan and presents different chitosan-base materials with antioxidant, antibacterial and inhibitory capacity against wound enzymes in the attempt of providing a potential way of down regulating proteolytic environment of chronic wounds.

2.7. Objectives

The overall aim of the present work was to develop new biomaterials capable to provide conditions to stimulate the chronic wound healing, as ideal candidates to further perform wound dressings. The specific research was defined in tree main targets:

- Modification of chitosan based platforms to balance **proteolytic activity of MMPs**
- Modification of chitosan based platforms to balance **of myeloperoxidases activity** and **oxidative environment**
- **Improve the antimicrobial behavior** of chitosan based platform

These modifications were expected to modulate the proteolytic and oxidative enzymes in chronic wounds and exert antimicrobial protection against wound bacterial infections.

To reach these goals the following specific objectives were define:

1. Functionalization of chitosan platform to improve the sequestering ability over metal dependent enzymes (MMPs) in chronic wounds (chapter 2). MMP inactivation can be induced by reagents which can induce the interaction with the zinc cation in the activation site, modifying MMP activity. This strategy of designing MMPs inhibitor is based on the chelating of Zn^{2+} that induces inhibition of MMPs. In order to bring to chitosan the ability to inhibit MMP, chitosan was functionalized with deferent thiol moieties. In addition the combination of -NH2 and -SH chemistry would allow for grafting of other active agents in further steps.

2. Identification and evaluation of potential active agents as a chronic wound healing promoter (chapter 3). Polyphenols from the medicinal plant *Hamamelis virginiana* (Witch hazel) were studied for this porpoise in an integrated approach simultaneously targeting antioxidant activity, inhibitory effect on MPO and collagenase

3. Development of a new method for permanent for functionalization of chitosan platform with active agents from *Hamamelis virginiana* extracts (chapter 4). This new method will allow obtaining a chitosan platform covalently modified with phenolic moieties. The new biopolymeric material will combine the chemistry of amino, thiol and phenolic groups and is expected to possess enhanced antioxidant capacity and antimicrobial effect.

4. Development of new hydrogel dressings for chronic wound application containing chitosan, gelatin, and natural phenolics, cross-linked by laccase reaction (chapter 5). This enzymatic method would allow to obtain bioactive and biostable materials with tunable

physicomechanical and functional properties. At the same time, polyphenols are expected to play a dual role in the hydrogel: i) “passive” – being a structural element, and ii) “active” – modifying the chronic wound environment by attenuating the wound enzymes, ROS activities, and the bacterial infection.

2.8. References

1. Thomas, D.R., *Issues and dilemmas in the prevention and treatment of pressure ulcers: a review.* J Gerontol A Biol Sci Med Sci, 2001. **56**(6): p. M328-40.
2. Berlowitz, D.R., et al., *Effect of pressure ulcers on the survival of long-term care residents.* J Gerontol A Biol Sci Med Sci, 1997. **52**(2): p. M106-10.
3. Allman, R., *Pressure ulcers among the elderly. Pressure ulcers among the elderly.*
4. Torra i Bou, J., J. Soldevilla Agreda, and J. Verdú Soriano, *Pressure Ulcers – Integrated Prevention and Treatment Approaches for an Old Problem Still Active in the 21st Century*. European Dermatology Review, 2007(ISSUE I).
5. Thoroddsen, A., *Pressure sore prevalence: a national survey.* J Clin Nurs, 1999. **8**(2): p. 170-9.
6. MacLellan, D.G., *Chronic wound management.* Autralian Presciber, 2000. **23**(1): p. 4.
7. Smith and Nephew. *Anual Review*. 2009 2009 [cited 2009 2009]; Available from: http://global.smith-nephew.com/cps/rde/xbcr/smithnephewls_master/annualreview.pdf
8. America, V.N.A.o., *Proteases and the Chronic Wound*. 2011, Visiting Nurse Associations of America.
9. Orgill, D. and C. Blanco, *Biomaterials for Treating Skin Loss*. 2009: CRC.
10. Steed, D.L., *The role of growth factors in wound healing.* The Surgical clinics of North America, 1997. **77**(3): p. 575-586.
11. Gosain, A. and L.A. DiPietro, *Aging and wound healing.* World J Surg, 2004. **28**(3): p. 321-6.
12. Broughton, G., 2nd, J.E. Janis, and C.E. Attinger, *The basic science of wound healing.* Plast Reconstr Surg, 2006. **117**(7 Suppl): p. 12S-34S.
13. Campos, A.C., A.K. Groth, and A.B. Branco, *Assessment and nutritional aspects of wound healing.* Curr Opin Clin Nutr Metab Care, 2008. **11**(3): p. 281-8.
14. Meszaros, A.J., J.S. Reichner, and J.E. Albina, *Macrophage-induced neutrophil apoptosis.* J Immunol, 2000. **165**(1): p. 435-41.
15. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.
16. Midwood, K.S., L.V. Williams, and J.E. Schwarzbauer, *Tissue repair and the dynamics of the extracellular matrix.* Int J Biochem Cell Biol, 2004. **36**(6): p. 1031-7.
17. Chang, H.Y., et al., *Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds.* PLoS Biol, 2004. **2**(2): p. E7.
18. Greenhalgh, D.G., *The role of apoptosis in wound healing.* Int J Biochem Cell Biol, 1998. **30**(9): p. 1019-30.
19. Clark, R.A., et al., *Fibronectin is produced by blood vessels in response to injury.* J Exp Med, 1982. **156**(2): p. 646-51.

20. Clark, R.A., et al., *Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization.* J Invest Dermatol, 1982. **79**(5): p. 264-9.

21. Singer, A.J. and R.A.F. Clark, *Cutaneous Wound Healing.* New England Journal of Medicine, 1999. **341**(10): p. 738-746.

22. Madlener, M., W.C. Parks, and S. Werner, *Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair.* Exp Cell Res, 1998. **242**(1): p. 201-10.

23. Roth, D., et al., *Plasmin Modulates Vascular Endothelial Growth Factor-A-Mediated Angiogenesis during Wound Repair.* The American Journal of Pathology, 2006. **168**(2): p. 670-684.

24. Singer, A.J. and R.A. Clark, *Cutaneous wound healing.* N Engl J Med, 1999. **341**(10): p. 738-46.

25. Grinnell, F. and M. Zhu, *Fibronectin degradation in chronic wounds depends on the relative levels of elastase, alpha1-proteinase inhibitor, and alpha2-macroglobulin.* J Invest Dermatol, 1996. **106**(2): p. 335-41.

26. Schultz, G.S., G. Ladwig, and A. Wysock, *Extracellular matrix: review of its roles in acute and chronic wounds* world wide wounds: http://www.worldwidewounds.com/2005/august/Schultz/Extrace-Matric-Acute-Chronic-Wounds.html, 2005.

27. Li, J., J. Chen, and R. Kirsner, *Pathophysiology of acute wound healing.* Clin Dermatol, 2007. **25**(1): p. 9-18.

28. Visse, R. and H. Nagase, *Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry.* Circ Res, 2003. **92**(8): p. 827-39.

29. Guo, S. and L.A. Dipietro, *Factors affecting wound healing.* J Dent Res, 2010. **89**(3): p. 219-29.

30. Sandeman, S.R., et al., *Human keratocyte migration into collagen gels declines with in vitro ageing.* Mech Ageing Dev, 2000. **119**(3): p. 149-57.

31. Swirski, F.K., et al., *Identification of splenic reservoir monocytes and their deployment to inflammatory sites.* Science, 2009. **325**(5940): p. 612-6.

32. Bryant, R.A. and D.P. Nix, *Acute & Chronic Wounds: Current Management Concepts*. 2010: Elsevier Mosby.

33. Evans, E., *Nutritional assessment in chronic wound care.* J Wound Ostomy Continence Nurs, 2005. **32**(5): p. 317-20.

34. Kane, D., *Chronic wound healing and chronic wound management. In: Chronic Wound Care: A Clinical Source Book for Heathcare Professionals* Vol. 4. 2007: HMP Communications.

35. Mustoe, T., *Understanding chronic wounds: a unifying hypothesis on their pathogenesis and implications for therapy.* The American Journal of Surgery, 2004. **187**(5, Supplement 1): p. S65-S70-S65-S70.

36. Peirce, S.M., T.C. Skalak, and G.T. Rodeheaver, *Ischemia-reperfusion injury in chronic pressure ulcer formation: A skin model in the rat.* Wound Repair and Regeneration, 2000. **8**(1): p. 68-76.

37. Sibbald, R.G., D.L. Krasner, and K.Y. Woo, *Pressure Ulcer Staging Revisited: Superficial Skin Changes & Deep Pressure Ulcer Framework©.* Advances in Skin & Wound Care, 2011. **24**(12): p. 571-580 10.1097/01.ASW.0000408467.26999.6d.

38. Lowthian, P.T., *Trauma and thrombosis in the pathogenesis of pressure ulcers.* Clin Dermatol, 2005. **23**(1): p. 116-23.

39. O'Neil, C.K., *Prevention and Treatment of Pressure Ulcers.* Journal of Pharmacy Practice, 2004. **17**(2): p. 137-148.

40. Trengove NJ, S.M., MacAuley S et al., *Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors.* Wound Repair and Regeneration, 1999. **7**: p. 442–452.

41. Yager, D.R. and B.C. Nwomeh, *The proteolytic environment of chronic wounds.* Wound Repair Regen, 1999. **7**(6): p. 433-41.

42. Davies, C.E., et al., *Use of molecular techniques to study microbial diversity in the skin: Chronic wounds reevaluated.* Wound Repair and Regeneration, 2001. **9**(5): p. 332-340.

43. Sen, C.K., et al., *Oxygen, oxidants, and antioxidants in wound healing: an emerging paradigm.* Ann N Y Acad Sci, 2002. **957**: p. 239-49.

44. James, T.J., et al., *Evidence of oxidative stress in chronic venous ulcers.* Wound Repair Regen, 2003. **11**(3): p. 172-6.

45. Nwomeh, B.C., et al., *Dynamics of the matrix metalloproteinases MMP-1 and MMP-8 in acute open human dermal wounds.* Wound Repair Regen, 1998. **6**(2): p. 127-34.

46. Nwomeh, B.C., et al., *MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers.* J Surg Res, 1999. **81**(2): p. 189-95.

47. Yager, D.R., et al., *Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids.* J Invest Dermatol, 1996. **107**(5): p. 743-8.

48. Wenk, J., et al., *Selective pick-up of increased iron by deferoxamine-coupled cellulose abrogates the iron-driven induction of matrix-degrading metalloproteinase 1 and lipid peroxidation in human dermal fibroblasts in vitro: a new dressing concept.* J Invest Dermatol, 2001. **116**(6): p. 833-9.

49. Kerstein, M., *Wound infection: Assessment and management.* 1996. **8**: p. 141–144.

50. Thomas S. (Cherry GW, H.K., Ryan TJ (eds)), *Sterile maggots and the preparation of the wound bed.* Proceedings of a symposium sponsored by the European Tissue Repair Society; 2000 Nov 24–25; Oxford UK. International Congress and Symposium Series 250. Royal Society of Medicine Press Limited,, 2001: p. 59–66.

51. Wiegand, C. and U.-C. Hipler, *Polymer-based Biomaterials as Dressings for Chronic Stagnating Wounds.* Macromolecular Symposia, 2010. **294**(2): p. 1-13.

52. Bader, D.L., et al., *Pressure Ulcer Research: Current And Future Perspectives*. 2005: Springer.

53. Mustoe, T.A., K. O'Shaughnessy, and O. Kloeters, *Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis.* Plast Reconstr Surg, 2006. **117**(7 Suppl): p. 35S-41S.

54. Rezkalla, S.H. and R.A. Kloner, *No-reflow phenomenon.* Circulation, 2002. **105**(5): p. 656-62.

55. Snyder, R.J., *Treatment of nonhealing ulcers with allografts.* Clin Dermatol, 2005. **23**(4): p. 388-395.

56. Alleva, R., et al., *α-Lipoic acid supplementation inhibits oxidative damage, accelerating chronic wound healing in patients undergoing hyperbaric oxygen therapy.* Biochemical and Biophysical Research Communications, 2005. **333**(2): p. 404-410.

57. Woessner, J.F., Jr., *The family of matrix metalloproteinases.* Ann N Y Acad Sci, 1994. **732**: p. 11-21.

58. Gibson, D., et al. (2009) *MMPs Made Easy*. **1**.

59. Gearing, A.J., et al., *Processing of tumour necrosis factor-alpha precursor by metalloproteinases.* Nature, 1994. **370**(6490): p. 555-7.

60. Stetler-Stevenson, W.G., *Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention.* J Clin Invest, 1999. **103**(9): p. 1237-41.

61. Pilcher, B.K., et al., *The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix.* J Cell Biol, 1997. **137**(6): p. 1445-57.

62. Parks, W.C., *Matrix metalloproteinases in repair.* Wound Repair Regen, 1999. **7**(6): p. 423-32.

63. Scott, K.A., E.J. Wood, and E.H. Karran, *A matrix metalloproteinase inhibitor which prevents fibroblast-mediated collagen lattice contraction.* FEBS Lett, 1998. **441**(1): p. 137-40.

64. Daniels, J.T., et al., *Matrix metalloproteinase inhibition modulates fibroblast-mediated matrix contraction and collagen production in vitro.* Invest Ophthalmol Vis Sci, 2003. **44**(3): p. 1104-10.

65. Page-McCaw, A., A.J. Ewald, and Z. Werb, *Matrix metalloproteinases and the regulation of tissue remodelling.* Nat Rev Mol Cell Biol, 2007. **8**(3): p. 221-33.

66. Ulrich, D., et al., *Matrix metalloproteinases and tissue inhibitors of metalloproteinases in patients with different types of scars and keloids.* J Plast Reconstr Aesthet Surg, 2010. **63**(6): p. 1015-21.

67. Palolahti, M., et al., *Proteolytic activity in leg ulcer exudate.* Experimental Dermatology, 1993. **2**(1): p. 29-37.

68. Harris, I.R., et al., *Cytokine and protease levels in healing and non-healing chronic venous leg ulcers.* Experimental Dermatology, 1995. **4**(6): p. 342-349.

69. Barrick, B., E.J. Campbell, and C.A. Owen, *Leukocyte proteinases in wound healing: roles in physiologic and pathologic processes.* Wound Repair and Regeneration, 1999. **7**(6): p. 410-422.

70. Weiss, S.J., *Tissue Destruction by Neutrophils.* New England Journal of Medicine, 1989. **320**(6): p. 365-376.

71. Wlaschek, M., et al., *Protease inhibitors protect growth factor activity in chronic wounds.* Br J Dermatol, 1997. **137**(4): p. 646.

72. Mannello, F. and V. Medda, *Nuclear localization of Matrix metalloproteinases.* Progress in Histochemistry and Cytochemistry, 2012. **47**(1): p. 27-58.

73. Klein, T. and R. Bischoff, *Physiology and pathophysiology of matrix metalloproteases.* Amino Acids, 2011. **41**(2): p. 271-90.

74. Van Wart, H.E. and H. Birkedal-Hansen, *The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family.* Proc Natl Acad Sci U S A, 1990. **87**(14): p. 5578-82.

75. Springman, E.B., et al., *Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation.* Proc Natl Acad Sci U S A, 1990. **87**(1): p. 364-8.

76. Bibi, N., *Elastase Responsive Hydrogel Dressing for Chronic Wounds*, in *School of Materials* 2010, University of Manchester, Faculty of Engineering

and Physical Sciences: Manchester. p. 249.

77. Klebanoff, S.J., *Myeloperoxidase.* Proc Assoc Am Physicians, 1999. **111**(5): p. 383-9.

78. Klebanoff, S.J., *Myeloperoxidase: friend and foe.* J Leukoc Biol, 2005. **77**(5): p. 598-625.

79. Dechert, T.A., et al., *Hyaluronan in human acute and chronic dermal wounds.* Wound Repair Regen, 2006. **14**(3): p. 252-8.

80. Harrison, J.E. and J. Schultz, *Studies on the chlorinating activity of myeloperoxidase.* J Biol Chem, 1976. **251**(5): p. 1371-4.

81. Daugherty, A., et al., *Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions.* J Clin Invest, 1994. **94**(1): p. 437-44.

82. Leeuwenburgh, C., et al., *Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques.* J Biol Chem, 1997. **272**(6): p. 3520-6.

83. Hazen, S.L. and J.W. Heinecke, *3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima.* J Clin Invest, 1997. **99**(9): p. 2075-81.

84. Leeuwenburgh, C., et al., *Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima.* J Biol Chem, 1997. **272**(3): p. 1433-6.

85. Carr, A.C., et al., *Relative reactivities of N-chloramines and hypochlorous acid with human plasma constituents.* Free Radic Biol Med, 2001. **30**(5): p. 526-36.

86. Peskin, A.V. and C.C. Winterbourn, *Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate.* Free Radic Biol Med, 2001. **30**(5): p. 572-9.

87. Fu, X., et al., *Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and*

atherosclerotic plaque rupture by myeloperoxidase. The Journal of Biological Chemistry, 2001. **276**(44): p. 41279-87.

88. Brew, K., D. Dinakarpandian, and H. Nagase, *Tissue inhibitors of metalloproteinases: evolution, structure and function.* Biochim Biophys Acta, 2000. **1477**(1-2): p. 267-83.

89. Bode, W., et al., *Insights into MMP-TIMP Interactions.* Annals of the New York Academy of Sciences, 1999. **878**(1): p. 73-91.

90. Fligiel, S.E., et al., *Matrix metalloproteinases and matrix metalloproteinase inhibitors in acute lung injury.* Hum Pathol, 2006. **37**(4): p. 422-30.

91. Wang, Y., et al., *Myeloperoxidase Inactivates TIMP-1 by Oxidizing Its N-terminal Cysteine Residue.* Journal of Biological Chemistry, 2007. **282**(44): p. 31826-31834.

92. Bucknall, T.E., *The effect of local infection upon wound healing: an experimental study.* Br J Surg, 1980. **67**(12): p. 851-5.

93. Thomson, P.D., *What is the role of bacteria in chronic wound exudates? In: Cherry G, Harding KG (eds). Management of wound exudate. Proceedings of a joint meeting of the European Wound Management Association and the European Tissue Repair Society. London, England. Churchill Communications Limited,.* 1997: p. 35–38.

94. Facklam, R. and W. J., *Streptococcus and elated catalase-negative gram-positive cocci. In: Balows A, Hausler W, Hermann K, et al. (eds). Manual of Clinical Microbiology, Fifth Edition. Washington, DC: American Society for Microbiology.* 1991: p. 238.

95. Bohnen, J., et al., *Pathogenicity of enterococcus in a rat model of fecal peritonitis. In: Program and abstracts of the Interscience Conference Antimicrobial Agents Chemotherapy. Abstract 276.* 1993.

96. Schuchat, A., *Group B streptococcus.* Lancet, 1999. **353**(9146): p. 51-6.

97. Dow, G., A. Browne, and R.G. Sibbald, *Infection in chronic wounds: controversies in diagnosis and treatment.* Ostomy Wound Manage, 1999. **45**(8): p. 23-7, 29-40; quiz 41-2.

98. Davey, M.E. and A. O'Toole G, *Microbial biofilms: from ecology to molecular genetics.* Microbiol Mol Biol Rev, 2000. **64**(4): p. 847-67.

99. Ladwig, G.P., et al., *Ratios of activated matrix metalloproteinase-9 to tissue inhibitor of matrix metalloproteinase-1 in wound fluids are inversely correlated with healing of pressure ulcers.* Wound Repair Regen, 2002. **10**(1): p. 26-37.

100. Warriner, R. and R. Burrell, *Infection and the chronic wound: a focus on silver.* Adv Skin Wound Care, 2005. **18 Suppl 1**: p. 2-12.

101. Wright, J.B., et al., *Early healing events in a porcine model of contaminated wounds: effects of nanocrystalline silver on matrix metalloproteinases, cell apoptosis, and healing.* Wound Repair Regen, 2002. **10**(3): p. 141-51.

102. Fazli, M., et al., *Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds.* Journal of Clinical Microbiology, 2009. **47**(12): p. 4084-9.

103. Costerton, J.W., et al., *Bacterial biofilms in nature and disease.* Annu Rev Microbiol, 1987. **41**: p. 435-64.

104. Drenkard, E., *Antimicrobial resistance of Pseudomonas aeruginosa biofilms.* Microbes Infect, 2003. **5**(13): p. 1213-9.

105. Gjodsbol, K., et al., *Multiple bacterial species reside in chronic wounds: a longitudinal study.* Int Wound J, 2006. **3**(3): p. 225-31.

106. Halbert, A.R., et al., *The effect of bacterial colonization on venous ulcer healing.* Australas J Dermatol, 1992. **33**(2): p. 75-80.

107. Davies, D., *Understanding biofilm resistance to antibacterial agents.* Nat Rev Drug Discov, 2003. **2**(2): p. 114-22.

108. Donlan, R.M. and J.W. Costerton, *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clin Microbiol Rev, 2002. **15**(2): p. 167-93.

109. Madsen, S.M., et al., *Bacterial colonization and healing of venous leg ulcers.* Apmis, 1996. **104**(12): p. 895-9.

110. Bjarnsholt, T., et al., *Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent.* Microbiology, 2005. **151**(Pt 2): p. 373-83.

111. Kirketerp-Moller, K., et al., *Distribution, organization, and ecology of bacteria in chronic wounds.* J Clin Microbiol, 2008. **46**(8): p. 2717-22.

112. Supuran, C.T., A. Scozzafava, and A. Mastrolorenzo, *Bacterial proteases: current therapeutic use and future prospects for the development of new antibiotics.* Expert Opinion on Therapeutic Patents, 2001. **11**(2): p. 221-259.

113. McCarty, S.M., et al., *The role of endogenous and exogenous enzymes in chronic wounds: A focus on the implications of aberrant levels of both host and bacterial proteases in wound healing.* Wound Repair and Regeneration, 2012. **20**(2): p. 125-136.

114. *Principles of best practice: Minimising pain at wound dressing-related procedures. A consensus document.* London: MEP Ltd, 2004.

115. Kim, B.-S., C.E. Baez, and A. Atala, *Biomaterials for tissue engineering.* World Journal of Urology, 2000. **18**(1): p. 2-9.

116. Mano, J.F., et al., *Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends.* Journal of The Royal Society Interface, 2007. **4**(17): p. 999-1030.

117. Jayakumar, R., et al., *Novel Chitin and Chitosan Materials in Wound Dressing*, in *Biomedical Engineering, Trends in Materials Science*, A.N. Laskovski, Editor. 2011.

118. Richardson, S.C., H.V. Kolbe, and R. Duncan, *Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA.* Int J Pharm, 1999. **178**(2): p. 231-43.

119. Tomihata, K. and Y. Ikada, *In vitro and in vivo degradation of films of chitin and its deacetylated derivatives.* Biomaterials, 1997. **18**(7): p. 567-75.

120. Lahiji, A., et al., *Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes.* J Biomed Mater Res, 2000. **51**(4): p. 586-95.

121. Muzzarelli, R.A., et al., *Osteoconduction exerted by methylpyrrolidinone chitosan used in dental surgery.* Biomaterials, 1993. **14**(1): p. 39-43.

122. Muzzarelli, R., et al., *Reconstruction of parodontal tissue with chitosan.* Biomaterials, 1989. **10**(9): p. 598-603.

123. Eser Elcin, A., Y.M. Elcin, and G.D. Pappas, *Neural tissue engineering: adrenal chromaffin cell attachment and viability on chitosan scaffolds.* Neurol Res, 1998. **20**(7): p. 648-54.

124. Nishioka, Y., et al., *A study of embolizing materials for chemo-embolization therapy of hepatocellular carcinoma: embolic effect of cisplatin albumin microspheres using chitin and chitosan in dogs, and changes of cisplatin content in blood and tissue.* Chem Pharm Bull (Tokyo), 1992. **40**(1): p. 267-8.

125. Elcin, Y.M., V. Dixit, and G. Gitnick, *Hepatocyte attachment on biodegradable modified chitosan membranes: in vitro evaluation for the development of liver organoids.* Artif Organs, 1998. **22**(10): p. 837-46.

126. Varum, K.M., et al., *In vitro degradation rates of partially N-acetylated chitosans in human serum.* Carbohydr Res, 1997. **299**(1-2): p. 99-101.

127. Kato, Y., H. Onishi, and Y. Machida, *Depolymerization of N-succinyl-chitosan by hydrochloric acid.* Carbohydr Res, 2002. **337**(6): p. 561-4.

128. Pangburn, S.H., P.V. Trescony, and J. Heller, *Lysozyme degradation of partially deacetylated chitin, its films and hydrogels.* Biomaterials, 1982. **3**(2): p. 105-8.

129. Hirano, S., H. Tsuchida, and N. Nagao, *N-acetylation in chitosan and the rate of its enzymic hydrolysis.* Biomaterials, 1989. **10**(8): p. 574-6.

130. Schipper, N.G., et al., *Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement.* Pharm Res, 1997. **14**(7): p. 923-9.

131. Fei Liu, X., et al., *Antibacterial action of chitosan and carboxymethylated chitosan.* Journal of Applied Polymer Science, 2001. **79**(7): p. 1324-1335.

132. Kong, M., et al., *Antimicrobial properties of chitosan and mode of action: a state of the art review.* Int J Food Microbiol, 2010. **144**(1): p. 51-63.

133. Muzzarelli, R.A.A., *Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone.* Carbohydrate Polymers, 2009. **76**(2): p. 167-182.

134. Kim, M.M. and S.K. Kim, *Chitooligosaccharides inhibit activation and expression of matrix metalloproteinase-2 in human dermal fibroblasts.* FEBS Lett, 2006. **580**(11): p. 2661-6.

135. Muzzarelli, R.A. and L. Sipos, *Chitosan for the collection from seawater of naturally occurring zinc, cadmium, lead and copper.* Talanta, 1971. **18**(9): p. 853-8.

136. Mendis, E., et al., *Carboxy derivatized glucosamine is a potent inhibitor of matrix metalloproteinase-9 in HT1080 cells.* Bioorganic & Medicinal Chemistry Letters, 2006. **16**(12): p. 3105-3110.

137. Van Ta, Q., M.M. Kim, and S.K. Kim, *Inhibitory effect of chitooligosaccharides on matrix metalloproteinase-9 in human fibrosarcoma cells (HT1080).* Mar Biotechnol (NY), 2006. **8**(6): p. 593-9.

138. Lin, Y.C., et al., *Chondroprotective effects of glucosamine involving the p38 MAPK and Akt signaling pathways.* Rheumatol Int, 2008. **28**(10): p. 1009-16.

139. Ong, S.Y., et al., *Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties.* Biomaterials, 2008. **29**(32): p. 4323-32.

140. Jayakumar, R., et al., *Biomaterials based on chitin and chitosan in wound dressing applications.* Biotechnol. Adv., 2011. **29**(3): p. 322-37.

141. Nishimura, K., et al., *Immunological activity of chitin and its derivatives.* Vaccine, 1984. **2**(1): p. 93-9.

142. Ueno, H., et al., *Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs.* Biomaterials, 1999. **20**(15): p. 1407-14.

143. Diegelmann, R.F., et al., *Analysis of the effects of chitosan on inflammation, angiogenesis, fibroplasia, and collagen deposition in polyvinyl alcohol sponge implants in rat wounds.* Wound Repair Regen, 1996. **4**(1): p. 48-52.

144. Kosaka, T., et al., *Effect of chitosan implantation on activation of canine macrophages and polymorphonuclear cells after surgical stress.* J Vet Med Sci, 1996. **58**(10): p. 963-7.

145. Yi, H., et al., *Biofabrication with chitosan.* Biomacromolecules, 2005. **6**(6): p. 2881-94.

Chapter 3

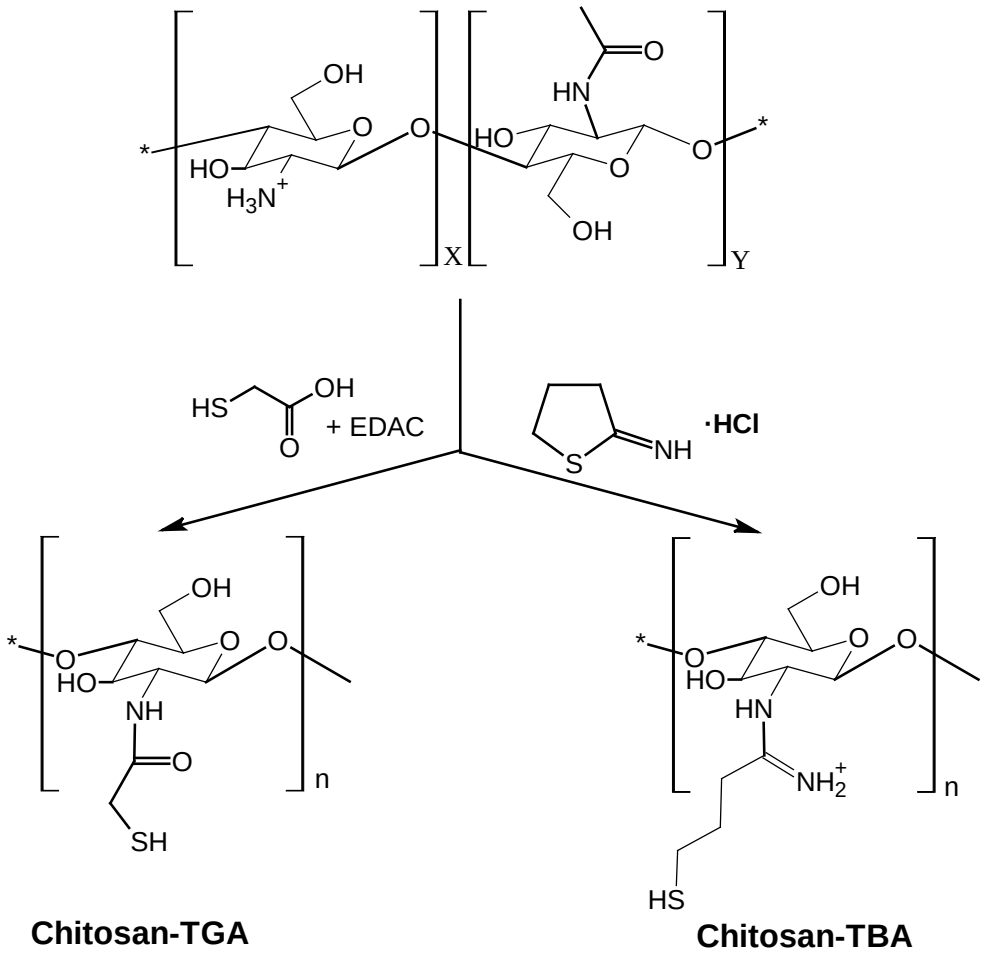
Modification of chitosan based platforms for inhibition of MMPs

3.1. Introduction

As is mentioned in the general introduction, the thiol group of cysteine in the propeptide interacts with a Zn^{2+} ion in the enzyme's active site. This interaction, [Zn^{2+}-cysteine], keeps the proMMP inactive [1, 2]. A mechanism known as the cysteine switch mobilizes proMMP's activation [3]. With the assistance of other MMPs, the second step involves cleaving the propeptide, which converts the enzyme into its active form. Activation pathways vary among MMPs (Figure 1.8). MMP inactivation can be induced by reagents which can induce the interaction with the zinc cation in the activation site, modifying MMP activity. The use of thiol zinc-binding groups in MMP inhibitor design is largely reported. This strategy of designing MMPs inhibitor is based on the chelating of Zn^{2+} that induces inhibition of MMPs

In order to bring to chitosan the ability to inhibit MMP, chitosan was functionalized with deferent thiol moieties. Therefore, various chitosan derivatives were synthesized varying the thiol molecule, thioglycolic acid and Trauts reagent (2-Iminothiolane HCl) and the amounts of immobilized thiol groups. Two types of thiolated chitosan were obtained: chitosan-cysteine conjugates, chitosan-thioglycolic (C-TGA) acid conjugates and chitosan-4-thio-butyl-amidine (C-TBA) conjugates. In all cases the primary amino group at the 2-position of the glucosamine subunits of chitosan is the main target for the immobilization of thiol groups (scheme 3.1). The resulting polymer conjugates were characterized by FTIR and the ability to inhibit collagenase activity was investigated.

These modifications aimed to improve the sequestering ability of chitosan over metal dependent enzymes (MMPs) in chronic wounds. In addition the combination of $-NH_2$ and -SH chemistry would allow for grafting of other active agents in further steps of this Thesis.



Scheme 3.1. Schematic representation of thiolation reaction of chitosan

3.2. Material and methods

3.2.1. Thiolated with thioglycolic acid using EDAC activation

Carboxylic acid group of thioglycolic acid (Sigma) reacts with the primary amino group of chitosan (KitoZyme, Belgium) mediated by a water soluble carbodiimide [4].

The immobilization of thiol groups on the surface of the chitosan was achieved by the covalent attachment of thioglycolic acid (TGA) (Sigma) to the primary amino groups of chitosan (KitoZyme, Belgium) via an amide bond. The coupling reaction was mediated by a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) [5] (Sigma).

Chitosan was dissolved in 1% HCl to obtain 1 % final concentration. A constant proportion 1:1 (w/w) chitosan:TGA was mixed,. Finally, different amounts EDAC were added. The pH was adjusted to 5 with 1M NaOH. After 3 h of incubation at room temperature under continuous stirring, the products were dialyzed 8 h against 5 mM HCl, two times for 8 h against 5 mM HCl containing 1 % NaCl, and finally 24 h against 1 mM HCl, to obtain pH5. Samples prepared in the same way, but omitting the addition EDAC, served as controls. Thereafter, the polymers were lyophilized and stored at -20 ºC under nitrogen.

3.2.2. Thiolated by 2-iminothiolane hydrochloride (Traut's reagent)

Thiolation with 2-iminothiolane hydrochloride (Traut's reagent) offers the advantage of a simple one step coupling reaction. The synthesis of chitosan-4-thio-butyl-amidine conjugate (chitosan–TBA conjugate) was carried out using a modification of the method developed by Bernkop-Schnurch et al. [6]. Chitosan was dissolved in 1 % HCl to obtain 1 % final concentration. The pH was adjusted to 6.5 with 2 M NaOH. Different amounts of 2-iminothiolane HCl (Traut's reagent) were added. After 24 h of incubation at room temperature under continuous stirring, the resulting chitosan–TBA conjugate was dialyzed 8 h against 5 mM HCl, two times for 8 h against 5 mM HCl containing 1 % NaCl, and finally 24 h against 1 mM HCl. A sample prepared in the same way but omitting the addition of 2-iminothiolane HCl served as a control. The polymers were liophylised thereafter. The degree of modification was monitored by a modified iodine titration method for determination of free -SH groups [7].

3.2.3. Determination of free SH groups on thiolated chitosan

The degree of thiol modification of chitosan was estimated by with Ellman's reagent [7]. Briefly, each conjugate was dissolved in demineralized water final concentration of 2% (m/v). To aliquots (250 µl) of the conjugate solutions, 250 µl of 0.5 M phosphate buffer pH 8.0 and 500 µl of Ellman's reagent (dissolved in 10 ml of 0.5 M phosphate buffer pH 8.0) were added. The reaction was allowed to proceed for 2 h at room temperature. Afterwards, the precipitated polymer was removed by centrifugation and 300 µl of the supernatant fluid was transferred to a microtitration plate. The absorbance was immediately measured at a wavelength of 450 nm with a microplate reader (Infinite M200, Tecan, Austria). Nonthiolated chitosan was used as a blank. The amount of thiol moieties was calculated from a standard curve elaborated at the same conditions with cysteine–HCl solutions.

3.2.4. FTIR analysis

Infrared spectra of the samples were collected over the 3500 - 800 cm-1 range using Perkin-Elmer Spectrum 100 (Perkin-Elmer, Massachusetts, USA) equipped with universal ATR sampling accessory, performing 50 scans for each spectrum..

3.2.5. Inhibition tests for proteolytic enzymes with thiolated chitosans

The inhibitory efficiency of chitosan and thiolated chitosan on proteolytic enzymes was assessed against serine protease Subtilisin A from Bacillus licheniformis (EC 3.4.21.14 from Novozymes) as a model for elastases, and collagenase with high purity from Clostridium Histolyticum (EC 3.4.24.3 from Sigma) as a model of MMPs. Subtilisin A activity was determined on casein substrate. Fluka 27669/27671 Collagenase Chromophore-Substrate Kit (for quantitative Collagenase-Determination) was used to measure collagenase activity. The specific collagenase substrate used was 4-Phenylazobenzyl-Pro-Leu-Gly-Pro-D-Arg-OH dehydrate.

3.3. Results and discussion

3.3.1. Characterization of functionalized chitosan

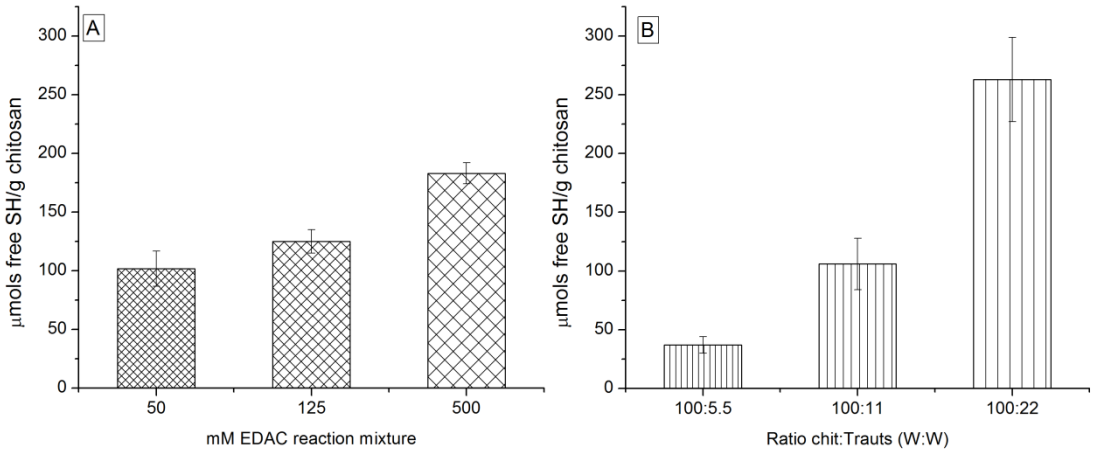


Figure 3.1. (A) Amount of free SH groups in TGA-Chit conjugate by the modified iodine titration method; chitosan concentration 1 % (w/v), Ratio chitosan:TGA 1:1 (w/w), at 25 ºC, pH 5.0; (B) Amount of free SH groups present in TBA-Chitosan conjugate determined by modified iodine titration method.

Good correlation between the increase of thiol groups and increase of Traut's reagent concentration or EDAC was observed (Figure 3.1.).

Thiolation was confirmed by FTIR (Figure 3.2.). The band at 1630 cm^{-1} attributable, in case of chitosan to C=O stretching of carboxylic acids and amides, increased in case of C-TGA and C-TBA due to the carbonyl (C=O) of thioglycolic acid and imino group (C=N) of Traut's reagent respectively [8] (Fig. 2.2). At the same time the band at 1203 cm^{-1} attributable to C-N stretching of amines increases after functionalization of chitosan, pointing out the formation of new C-N bounds. In chitosan spectra it is observable a band at 1593 cm^{-1} which corresponds to NH^{+2} salts [8] that disappears after functionalization. These observations confirm functionalization of chitosan with thioglycolic acid and Traut's reagent though the free amino groups.

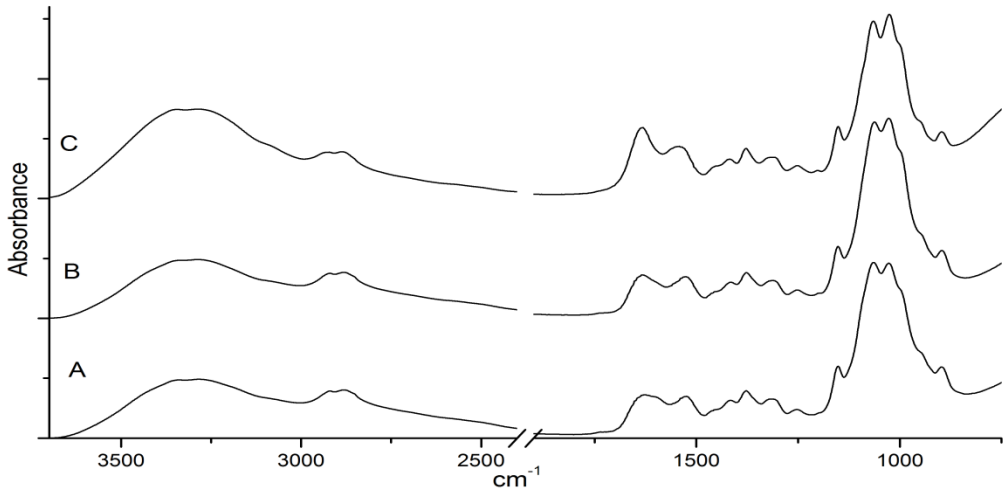


Figure 3.2. FTIR spectra of A) unmodified and thiolated chitosan, B) C-TGA and C) C-TBA.

3.3.2. Inhibition of collagenase

In this study, both C-TBA and C-TBA platform improved the inhibition of collagenase activity when compared with unmodified chitosan, suggesting that thiol moieties are involve in the process of inhibition of collagenase as was expected.

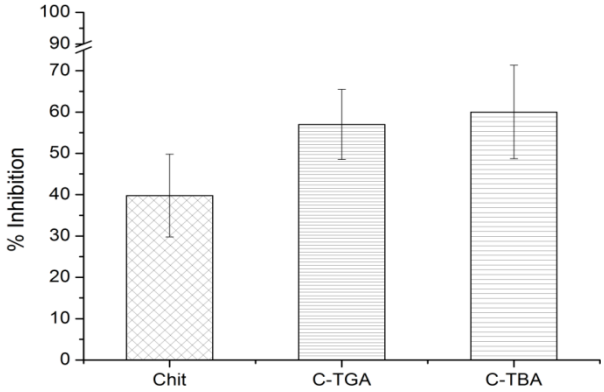


Figure 3.3. Inhibition of collagenase after 2 h at 25 ºC in TRIS-HCl buffer pH 7.1 with chitosan and thiolated chitosan conjugates; C-TGA (thioglycolic acid : EDAC 1:2 w/w), chi-TBA (chitosan : Traut´s 5:2 w/w)

3.4. Conclusions

The functionalization of chitosan with thiol moieties implements chitosan with inhibitory properties against collagenase. This improvement makes this functionalized chitosan good candidates as a platforms to configure wound dressings. In addition the combination of $-NH_2$ and -SH chemistry would allow for grafting of other active agents.

3.5. References

1. Springman, E.B., et al., *Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation.* Proc Natl Acad Sci U S A, 1990. **87**(1): p. 364-8.

2. Van Wart, H.E. and H. Birkedal-Hansen, *The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family.* Proc Natl Acad Sci U S A, 1990. **87**(14): p. 5578-82.

3. Nagase, H. and J.F. Woessner, Jr., *Matrix metalloproteinases.* J Biol Chem, 1999. **274**(31): p. 21491-4.

4. Bernkop-Schnurch, A., M. Hornof, and D. Guggi, *Thiolated chitosans.* Eur. J. Pharm. Biopharm., 2004. **57**(1): p. 9-17.

5. Hornof, M.D., C.E. Kast, and A. Bernkop-Schnurch, *In vitro evaluation of the viscoelastic properties of chitosan-thioglycolic acid conjugates.* Eur J Pharm Biopharm, 2003. **55**(2): p. 185-90.

6. Bernkop-Schnurch, A., C.E. Kast, and D. Guggi, *Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems.* J Control Release, 2003. **93**(2): p. 95-103.

7. Bravo-Osuna, I., et al., *Characterization of chitosan thiolation and application to thiol quantification onto nanoparticle surface.* Int. J. Pharm., 2007. **340**(1-2): p. 173-81.

8. Coates, J., *Interpretation of Infrared Spectra, A Practical Approach.* Encyclopedia of Analytical Chemistry.

Chapter 4

Plant polyphenols as scavengers of reactive oxygen species and inhibitors of deleterious chronic wound enzymes

Publications derived from this work: Díaz-González, M., Rocasalbas, G., Francesko, A., Touriño, S., Torres, J. L., & Tzanov, T. (2012). Inhibition of deleterious chronic wound enzymes with plant polyphenols. Biocat. Biotrans., 30(1), 102-110.

4.1. Introduction

Chronic wound is a complex environment characterized with the simultaneous interaction of elevated levels of neutrophils, neutrophil-derived proteolytic enzymes [1] and reactive oxygen species (ROS) [2]. MMPs, degrade components of the extracellular matrix such as elastin and collagen, and growth factors formed in the wound site, thereby impairing the healing [3, 4]. Collagenase is a matrix metalloproteinase largely present in chronic wounds [5] that specifically recognizes and cleaves collagen. In healthy tissues these proteases are counteracted by their natural inhibitors [6], while in chronic wounds the ratio of proteases to their natural inhibitors is biased in favor of proteases, so that most of the enzymes are uninhibited.

The other major factor governing the protease–antiprotease balance is the generation of hypochlorous acid (HOCl), the most powerful neutrophil oxidant [7], by MPO. The cytotoxicity of this reaction allows the killing of the invading microbial pathogens. However, the HOCl generated also reacts with most biological molecules, including the protease inhibitors, thereby promoting proteolytic damage in chronic wounds.

It could be expected that chronic wounds would start to heal as soon as the physiological balance, similar to that in acute wounds, is restored. This implies reducing the concentration/activity of protein degrading enzymes together with scavenging and/or inhibition of ROS in a controlled manner to encourage the healing process. Polyphenolic compounds present as secondary metabolites in plants have recently attracted much attention due to their curative properties. Their structures vary from simple molecules, such as phenolic acids, to highly polymerized compounds. The degree of polymerization and the chemical nature of the repetitive units constitute important structural features related to their physicochemical and biological properties. The primary effect of plant polyphenols is believed to reside in their antioxidant capacity and scavenging activity over both free radical and non-radical reactive species [8], metal-chelating capability [9] and inhibitory activity over radical-generating enzymes [10]. Plant polyphenols have been also reported to have anti-inflammatory [11], anti-microbial [12], immunomodulatory [13] and wound healing promoting properties [14] mostly explained by their antioxidant activity [15]. All of these benefits provided by the plant polyphenols could be employed in chronic wound management.

Polyphenols from bark, twigs and leaf extracts from the medicinal plant *Hamamelis virginiana* (Witch hazel) are widely used as components of skin care products and in dermatological treatment of sun burn, irritated skin etc. [16]. The aim of this work was to evaluate the potential of a polyphenol extract from *H. virginiana* as a chronic wound healing promoter in an

integrated approach simultaneously targeting antioxidant activity, inhibitory effect on MPO chlorinating activity and collagenase inhibition.

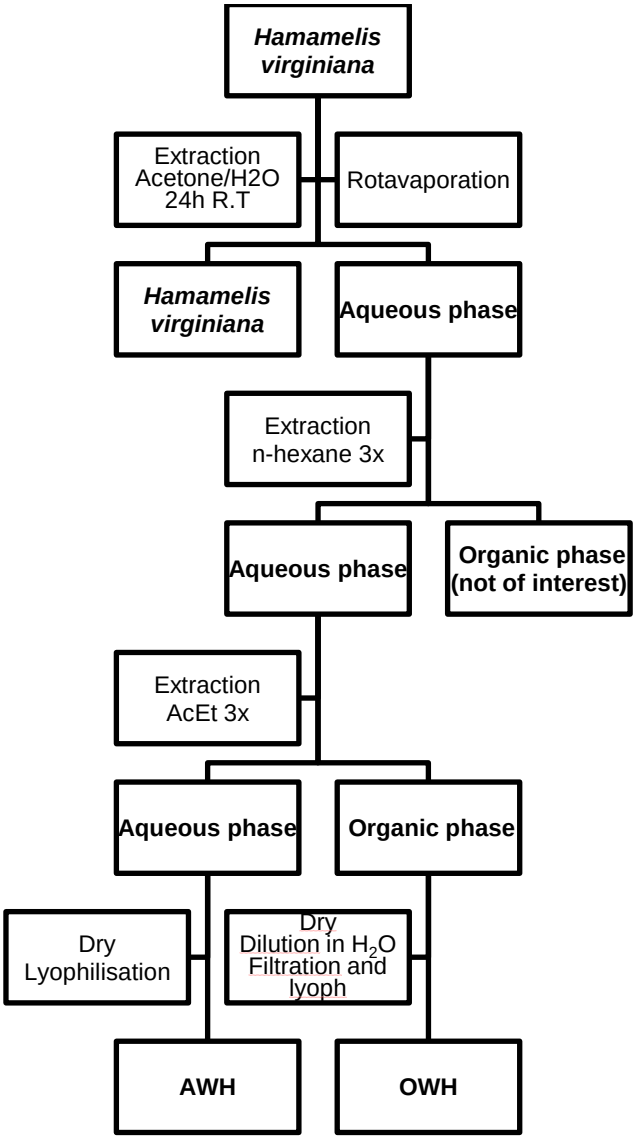
4.2. Materials and methods

4.2.1. Materials

Tris(hydroxymethyl)aminomethane, cysteamine hy drochloride, tricine, taurine, acetone, acetic acid, ascorbic acid, Na_2CO_3, NaOCl (13%), KI, NaCl, NaI, $CaCl_2$, NaOH, HCl, $Zn(NO_3)_2 \times 6H_2O$, 2,2-diphenyl-2-picrylhydrazyl hydrate(DPPH), H_2O_2 (30%), Folin–Ciocalteu's phenol reagent, 3,3′,5,5′-tetramethylbenzidine (TMB), (±)6-Hydroxy-2,5,7, 8-tetramethylchromane-2-carboxylic acid (Trolox), dimethyl sulfoxide (DMSO), (ethylenedinitrilo)tetra acetic acid (EDTA) and N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala (FALGPA); HPLC standards: (−) +epicatechin +(EC), (+) +catechin (c), (+) +gallocatechin (GC), (−)epigallocatechin (EGC), (+)epigallocatechin 3+ O-gallate (EGCG), gallic acid (GA), methyl gallate (MG) and hamamelitannin (HT); collagenase from *Clostridium histolyticum* (EC 3.4.24.3) and catalase (EC 1.11.1.6) from bovine liver were purchased from Sigma–Aldrich (Spain). Analytical grade MeOH and Ethanol 96% were obtained from Panreac (Spain). Acetonitrile HPLC grade and trifluoroacetic acid (TFA) were purchased from Fluka BioChemika (Spain). Myelo peroxidase from human polymorphonuclear leukocytes was provided by Planta Natural Products (Austria). All chemicals used in this work were of the highest grade commercially available. Ultra-pure water obtained with a Milli-Q plus 185 from Milipore Ibérica S.A. (Spain) was used for all solutions.

4.2.2. Preparation of Hamamelis virginiana extracts

Witch hazel (*Hamamelis virginiana*) was provided by Martin Bauer GmbH (Alveslohe, Germany). The phenolic extracts were prepared according to a described methodology [16-19] illustrated in Scheme 4.1. Briefly, chopped stems of *H. virginiana* were incubated with an acetone/water mixture for a period of 24 h at room temperature. The solid was filtered off, and the acetone was evaporated at reduced pressure. The remaining solution was defatted with *n*-hexane, and the oligomeric fraction was extracted with ethyl acetate. The aqueous dry fraction (AWH) was obtained after freeze-drying the aqueous solution. The organic phase was drying under vacuum, the resulting pellet was dissolved in deionized water, and the solution filtered through a porous plate. The organic dry fraction (OWH) was finally obtained by freeze-drying.



Scheme 4.1. Scheme of extraction of polyphenolic fractions from *Hamamelis virginiana*

3.2.3. Characterization by Thiolysis with Cysteamine and RP-HPLC

The size and composition of the phenolic compounds and the procyanidins within the fractions were estimated from the HPLC analysis after thioacidolytic depolymerization in the presence of cysteamine as previously described [20, 21]. The following conditions were used: loading volume 40 μL, 10 μg; elution, (A) 0.1% (v/v) aqueous trifluoroacetic acid (TFA) and (B)0.08% (v/v) TFA in water/acetonitrile 1:4, gradient 12–30% B over 30 min at a flow rate of 1 mL/min. Reverse phase high performance liquid chromatography (RP-HPLC) analysis was conducted on a Hitachi (San Jose, CA) Lachrom Elite HPLC system, equipped with diode array detector (DAD) and fitted with an analytical column (25 cm × 0.4 cm i.d., 100 Å, 5 μm particle size)

Kromasil C18 (Teknokroma, Spain). Detection was performed from 210 to 380 nm and acquisitions were examined using EZChrom Elite 3.1.3 from Scientific Software Inc. (Pleasanton, USA). Data were acquired in triplicate. The amount of the released moieties was calculated from the peak areas and compared to the calibration curves obtained with the pure standards. Terminal units: (+) GC, (-)- EGC, (+) C, (-)- EC, (-) EGCG and (-) ECG and (-) ECG; extension units: cysteamine (Cya)-C, Cya-EC, Cya-EGC, Cya-EGCG, and Cya-ECG. The mean degree of polymerization (mDP) was estimated by dividing the molar amounts of total units by the molar amounts of the terminal units.

3.2.4. The total phenolic content

The total phenolic content in the extracts were estimated as gallic acid equivalents, according to the Folin–Ciocalteu's method [22]. Initially, 50 μL of sample (25 mg/mL) were mixed with 1.2 mL of H_2O and 0.25 mL of undiluted Folin–Ciocalteu's reagent. After 1 min, 0.75 mL of 20% aqueous Na_2CO_3 were added, and the volume made up to 5 mL with H_2O. After 2 h of incubation at room temperature, the absorbance was measured at 760 nm in a microplate reader (Infinite M200, Tecan, Austria). Gallic acid standards were used to build the calibration curve and estimate the phenolic content as gallic acid equivalents. The control samples contain all the reaction reagents except the extract. The results are the mean values of three measurements

3.2.5. Electrochemical measurements

Electrochemical measurements were carried out using a μAutolab Type III EcoChemie, (Metrohm, The Netherlands) potentiostat/galvanostat controlled by Autolab GPES software version 4.9. All the experiments were carried out in a 20 mL Metrohm (Switzerland) cell with a three-electrode configuration. The working electrode was a glassy carbon (GCE) with a surface diameter of 3 mm (Metrohm). The counter and reference electrodes were platinum and Ag/AgCl electrodes from Metrohm, respectively. The renewal of the glassy carbon surface was achieved by polishing with 1.0 and 0.3 μm alpha-alumina (Micropolish, Buehler, USA) on a microcloth polishing pad (Buehler), followed by washing in an ultrasonic Selecta bath for 2 min. For the experiment with MPO a 5 μL sample of a 2 μM enzyme solution was dropped onto the polished surface of the GCE and allowed to dry for 15 min at room temperature.

3.2.6. Characterization of antioxidant activity of the fraction in vitro

Radical scavenging activity of the OWH fraction was determined by measuring the decrease in absorbance of $DPPH^{\bullet}$ radical at 515 nm [15]. Different concentrations of the OWH fraction were incubated with 3 mL of a $DPPH^{\bullet}$ (6×10^{-5} M) in MeOH at room temperature in dark for 15 min. The experiment was carried out in triplicate and the results expressed as %-inhibition of DPPH. Trolox, a water-soluble analogue of α-tocopherol (vitamin E), was used as reference compound. Antioxidant activity was evaluated as the concentration required to inhibit 50% of DPPH radical formation (IC_{50}). Non-radical scavenging activity was tested using hypochlorous acid. HOCl was measured by chlorination of taurine as previously described by [23]. The method quantifies the amount of taurine chloramine produced using iodide to catalyze the oxidation of TMB. The reaction mixture (300 µL) was prepared in 50 mM PBS pH 6.5 containing 150 mM NaCl, 5 mM taurine, NaOCl (50 µM in HOCl) and different concentrations of the extract. Finally, 75 µL of the detection reagent containing TMB (2 mM) and NaI (100 µM), in 400 mM acetate buffer pH 5.4, 10% DMSO, were added to the reaction mixture. After 5 min, the quantity of taurine chloramine generated was measured by recording the absorbance at 650 nm. Ascorbic acid as a potent HOCl scavenger was used as a reference. HOCl concentration from NaOCl solutions was determined by iodometry with a 20 mM KI solution. Iodine formed was measured at 350 nm and the acid concentration calculated using an extinction coefficient of 22 900 M^{-1} cm^{-1} [24]. All determinations were made in triplicate and expressed as %-inhibition of taurine chlorination. The concentrations required to inhibit 50% of taurine chlorination were also determined (IC_{50}).

3.2.7. Myeloperoxidase assay

Peroxidase activity of MPO was evaluated using guaiacol as substrate [25]. The inhibitory effect of plant extracts on MPO peroxidase activity was measured in a solution containing MPO (13 nM), guaiacol (10 mM) and H_2O_2 (100 µM) in 50 mM PBS pH 6.6. After 1 min of enzymatic reaction the change in absorbance at 470 nm was recorded. Inhibition of myeloperoxidase chlorinating activity was assessed as previously described for the scavenging assay. The method quantifies the amount of taurine chloramine produced by the $MPO/H_2O_2/Cl^-$ .The reaction mixture (300 µL) contained the following reagents, at final concentrations stated between brackets, in 50 mM PBS pH 6.5, 150 mM NaCl, 5 mM taurine: MPO (13 nM), H_2O_2 (100 µM) and OWH extract (0–50 µg/mL). After 2 min at 37°C, the enzymatic reaction was stopped by addition of 10 µL of 1 mg/mL catalase solution. Finally, 75 µL of the detection reagent (2 mM TMB, 100 µM NaI in 400 mM acetate buffer, pH 5.4, 10% DMSO) were added

to the reaction mixture. After 5 min the quantity of taurine chloramine generated during the enzymatic reaction was measured by recording the absorbance at 650 nm. MPO concentration was selected in order to produce about 60 μM of HOCl during the 2 min of the enzymatic reaction. All the assays were done in triplicate and the results expressed as the concentrations required to inhibit 50% production of HOCl by MPO (IC_{50}).

3.2.8. Zn^2-binding assay

The Zn^{2+}-binding assays were performed in 50 mM Tricine, 100 mM $CaCl_2$, 400 mM NaCl, pH 7.5 using $Zn(NO_3)_2 \times 6H_2O$. EDTA solutions were prepared in 0.1 M Tris-HCl, pH 8. The concentration of the extract used for the spectrophotometric studies was 100 μg/mL. The direct determination of zinc in the electrochemical assays was carried out by differential pulse anodic stripping voltammetry (DPASV) on a bare GCE. The zinc ions (25 μM) were first deposited by reduction at -1.5 V during 10 s and then detected by scanning the potential from 1.5 to 0.9 V.

3.2.9. Collagenase inhibition

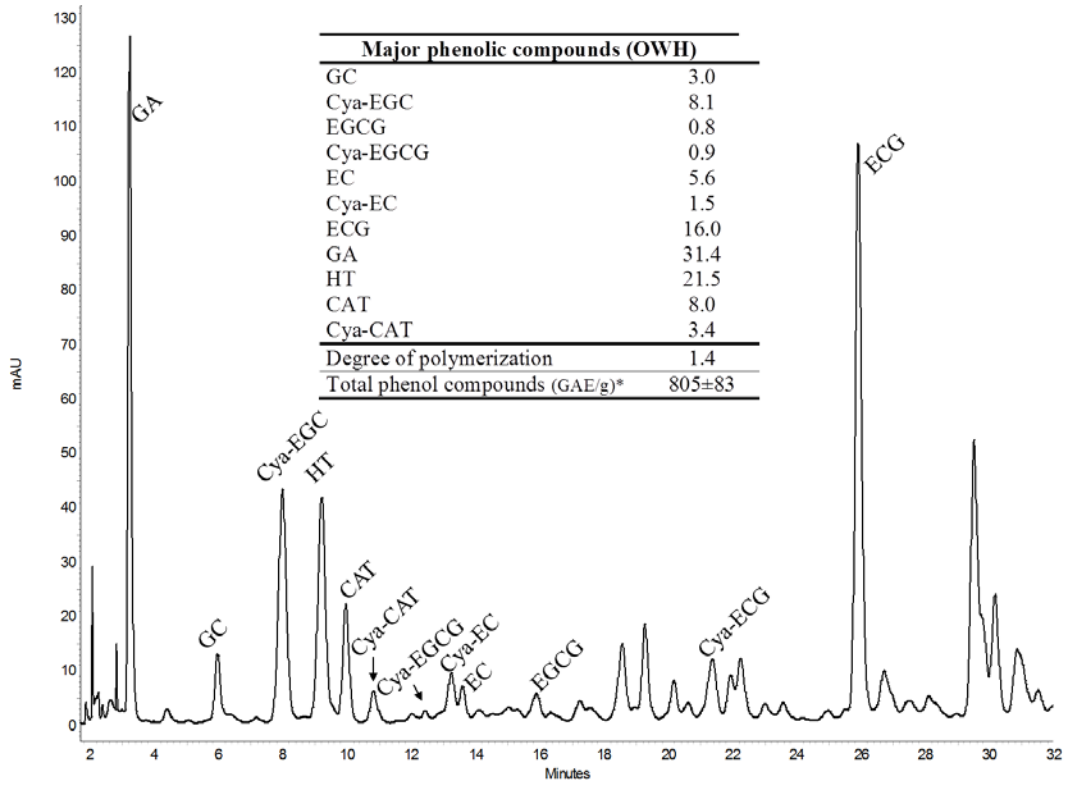
The collagenase activity was measured as described by [26], using FALGPA as substrate. The hydrolysis of FALGPA (1 mM) in 50 mM Tricine, 100 mM $CaCl_2$, 400 mM NaCl, pH 7.5 was monitored at 345 nm over 5 min. Collagenase final concentration was 0.2 μg/mL. Inhibition studies were carried out by adding different concentrations of the extract to the collagenase solution and incubating for 1 h at 25°C. The reaction was then initiated by addition of FALGPA substrate. All the assays were done in triplicate and the results expressed as the concentration required to inhibit 50% hydrolysis of FALGPA by collagenase (IC_{50}).

To determine the kinetics of collagenase inhibition, the experiment above was repeated for different collagenase concentrations. The kinetic parameters were calculated from Eisenthal-Cornish-Bowden plot and secondary plots of Kmapp/Vmaxapp vs [I] and 1/Vapp vs [I].

4.3. Results and discussion

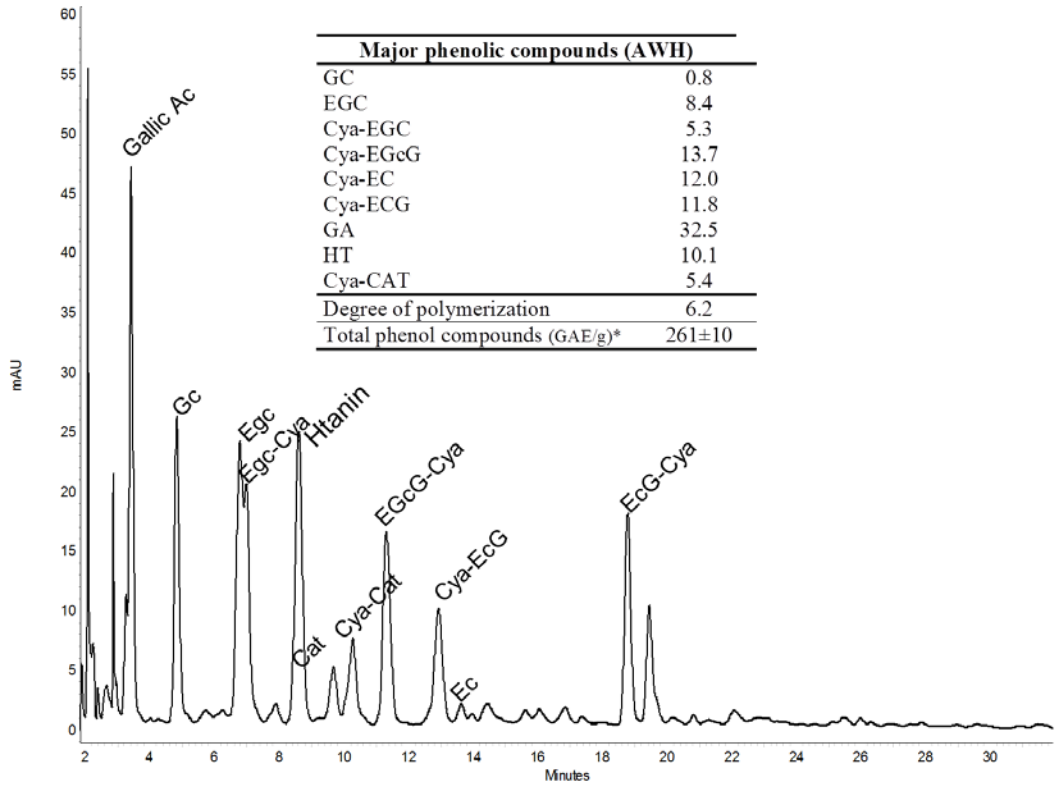
4.3.1. Characterization of H. virginiana fraction

The total phenol content of the OWH and AWH fraction was determined by the Folin–Ciocalteu method, and expressed as milligrams of gallic acid per gram of extract (EGA/g). The mean degree of polymerization and galloylation of the condensed tannin portion (monomers + proanthocyanidins) of both fractions was estimated by thioacidolysis in the presence of cysteamine. The fraction contained flavanol (catechin) monomers, proanthocyanidins and hydrolysable tannins (Figure 4.1 and 4.2). The structures of the major compounds found in the *H. virginiana* fraction are depicted in Table 4.1. OWH was rich in monomeric flavanol units, showing degree of polymerization (1.4) meanwhile AWH was rich in oligomeric structures (degree of polymerization 6.2) similar to that previously reported [21].



Major phenolic compounds (OWH)	
GC	3.0
Cya-EGC	8.1
EGCG	0.8
Cya-EGCG	0.9
EC	5.6
Cya-EC	1.5
ECG	16.0
GA	31.4
HT	21.5
CAT	8.0
Cya-CAT	3.4
Degree of polymerization	1.4
Total phenol compounds (GAE/g)*	805±83

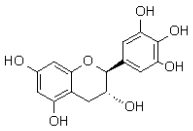
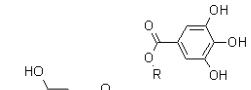
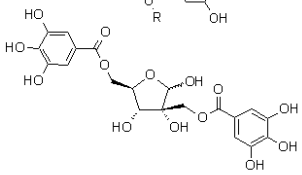
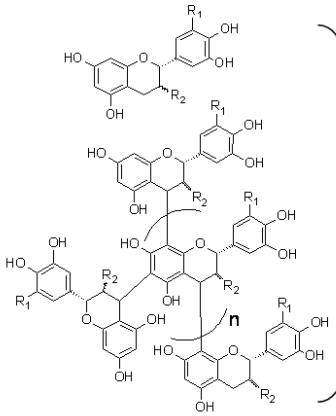
Figure 4.1. Chromatogram of OWH fraction after thiolation with cysteamine The characterization was performed in Lachrom Elite HPLC, photodiode array UV detector, column Kromasil C18 Teknokroma, Load, 40 µL, 10 µg; elution, [A] 0.1% (v/v) aqueous TFA and [B] 0.08% (v/v) TFA in water/CH3CN 1:4, gradient 12-30% B over 30 min, flow rate 1mL/min. The inset shows the percentage of the main components of the plant extract. * GAE (gallic acid equivalents)



Major phenolic compounds (AWH)	
GC	0.8
EGC	8.4
Cya-EGC	5.3
Cya-EGcG	13.7
Cya-EC	12.0
Cya-ECG	11.8
GA	32.5
HT	10.1
Cya-CAT	5.4
Degree of polymerization	6.2
Total phenol compounds (GAE/g)*	261±10

Figure 4.2. Chromatogram of AWH fraction after thiolation with cysteamine The characterization was performed in Lachrom Elite HPLC, photodiode array UV detector, column Kromasil C18 Teknokroma, Load, 40 µL, 10 µg; elution, [A] 0.1% (v/v) aqueous TFA and [B] 0.08% (v/v) TFA in water/CH_3CN 1:4, gradient 12-30% B over 30 min, flow rate 1mL/min. The inset shows the percentage of the main components of the plant extract. * GAE (gallic acid equivalents)

Table 4.1. Structures of the main polyphenolic compounds found in the OWH and AWH fraction

Chemical structure	Functional groups	Compounds
		GC = Gallocatechin
	R= H R= CH_3	GA = Gallic Acid MG = Methyl gallate
		HT = Hamamelitanin
	R_1= OH R_2=OH R_1= OH R_2=GA R_1= H R_2=OH R_1= H R_2=GA R_1= H R_2=H	EGC = Epigallocatechin EGCG = Epigallocatechin gallate EC = Epicatechin ECG = Epicatechin gallate CAT = Catechin

As the composition of OWH was richer in phenolics, it was chosen to perform the followed study for further chitosan modification described in Chapter 6, meanwhile AWH fraction as it was richer in oligomeric phenolics it was chosen to chitosan modification described in Chapter 5.

The total antioxidant capacity of the OWH fraction was evaluated in terms of scavenging effect over DPPH radical and HOCl acid (Figure 3.3). The results indicate that the reference compound Trolox was a more efficient (IC_{50} = 6.6 ± 0.3 μg/mL) DPPH radical scavenger than the OWH fraction (IC_{50} = 10.7 ± 0.7 μg/mL). However, both OWH and Trolox at concentrations of 20 μg/mL reached 90% of DPPH inhibition.

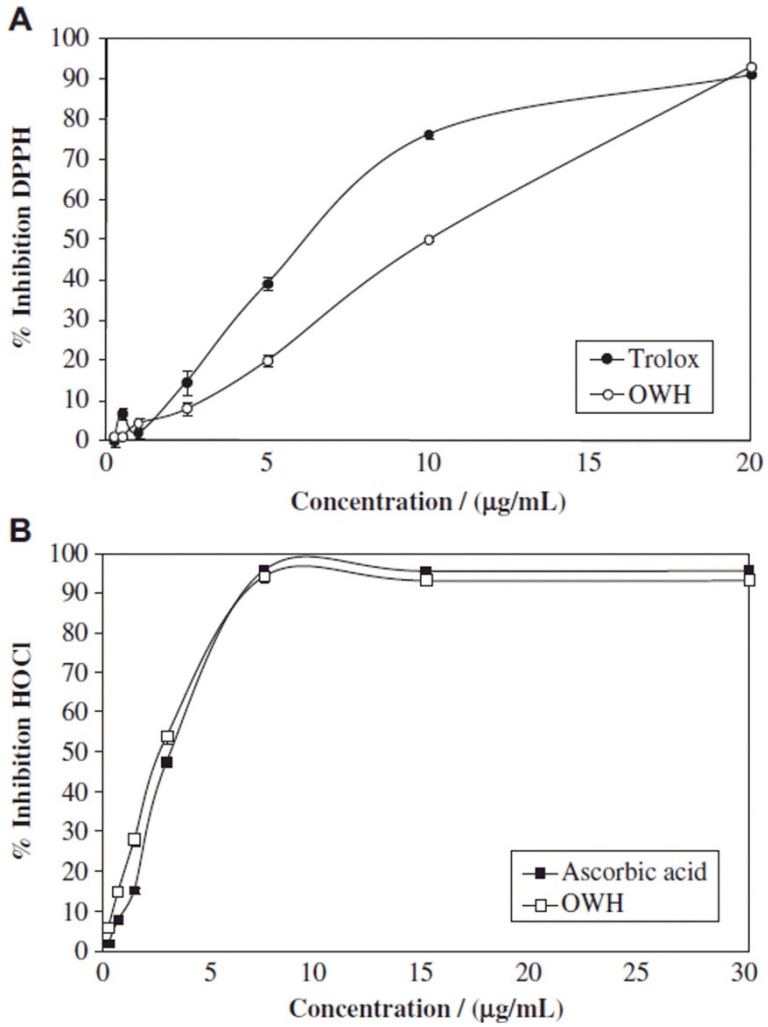


Figure 4.3. Antioxidant activity of OWH fraction in terms of: (A) free-radical and (B) non-radical scavenging activity.

The hypochlorous acid (50 µM) scavenging activity of OWH was slightly higher than that of ascorbic acid used as a reference, with IC_{50} values of 2.7 ± 0.1 and 3.3 ± 0.1 µg/mL respectively. These results clearly indicate that OWH fraction is an efficient scavenger of radical and non-radical reactive species.

4.3.2. Myeloperoxidase inhibition

A simplified mechanism of MPO catalytic cycle is shown in Figure 4.4. In the presence of H_2O_2, the ferric (FeIII)-form of MPO is oxidized by a two-electron equivalent to the redox intermediate compound I. This compound is a strong oxidant mediating both one- and two-electron oxidation reactions. Depending on the substrate availability, the enzyme follows either the halogenation or the peroxidase cycle. The halogenation cycle is a direct two-electron

reduction of compound I to the native form of the enzyme. In the peroxidase cycle, compound I is reduced in a two-step one-electron sequential reaction via formation of compound II. Many inorganic and organic substrates have been found to act as electron donors for compounds I and II. The principal reaction catalysed by MPO under physiological conditions is the oxidation of Cl^- to the corresponding hypochlorous acid. This acid is a strong chlorinating oxidant and is able to initiate modification reactions affecting lipids, DNA and (lipo)proteins. The inhibition of MPO chlorination activity could be envisaged at different levels including: (i) diversion of MPO from the chlorination cycle, and (ii) application of HOCl scavengers. Thus, in addition to the HOCl-scavenging ability of the OWH, the phenolic extract should be evaluated as a substrate for the peroxidase cycle of MPO as well. As a high concentration of scavengers is usually required to be effective, diversion of the enzyme from the chlorination cycle, suppressing excessive HOCl production, might be a suitable alternative. Furthermore, different phenolic compounds have already been reported to be MPO peroxidase substrates [27].

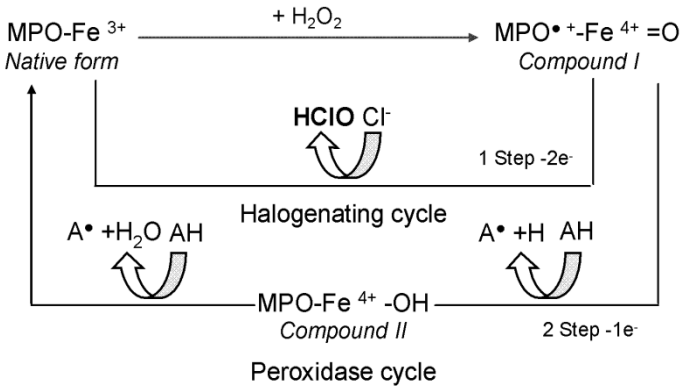


Figure 4.4. MPO catalytic cycle.

Figure 4.5A shows cyclic voltammograms of OWH in the presence and absence of MPO. The irreversible oxidation peak (E_{pa}) at 250 mV in the cyclic voltammogram of OWH alone is related to the oxidation of phenols present in the extract. An important decrease in the anodic peak current was observed in the presence of MPO. This was due to the catalytic but not electrodic oxidation of the polyphenols from the OWH fraction. If these compounds were oxidized by MPO, a decrease in the oxidation current would be expected.

In order to verify whether OWH polyphenols are peroxidase substrates for MPO, their inhibitory effect on MPO peroxidase activity using guaiacol as a substrate was studied (Figure 4.5B). The decrease of the absorbance at 470 nm, where guaiacol oxidation products show their

maximum absorption in the presence of different concentrations of polyphenols (IC_{50} = 7.6 ± 0.4 μg/mL), confirmed the ability of the OWH extract to act as a peroxidase substrate for MPO.

Once the role of the OWH extract in the peroxidase cycle was established, the effect of this extract on MPO chlorination was studied. The halogenation activity of MPO was determined by measuring the chlorination of taurine with a MPO/H_2O_2/Cl^- system. For this assay the MPO concentration was calculated in order to produce about 60 μM of HOCl after 2 min of enzymatic reaction in the presence of 150 mM Cl^-. The OWH extract inhibited taurine chlorination mediated by the MPO/H_2O_2/Cl^- system in a dose- dependent manner (data not shown), giving 50% inhibition (IC_{50}) for 1.3 ± 0.1 μg/mL of the extract. This IC_{50} value, lower than the value previously calculated for HOCl-scavenging activity, confirms that plant extracts with moderate scavenging activity were able to inhibit the HOCl production by MPO.

To understand the mechanism by which plant polyphenols were able to suppress the generation of HOCl, the changes in the absorption spectrum of MPO during enzyme turnover in the presence or absence of the extract were studied. Changes in the MPO spectrum can be followed by monitoring the absorbance between 390 and 600 nm where both the native form of the enzyme (430 nm) and compound II (456 nm) have their maximum absorption. As shown in Figure 3.4C, the native form of MPO (peak at 430 nm) was observed when the substrate was a chloride anion, since the redox intermediate compound-I reacts rapidly with the chloride-generating HOCl. However, the addition of a competitor substrate such as OWH extract to the reaction mixture promoted the formation of compound II (peak at 456 nm), which is unable to catalyze the production of HOCl. Therefore, the polyphenols from *H. virginiana* are not only scavengers of HOCl but can also serve as substrates in the MPO peroxidase cycle, thereby diverting the enzyme from its chlorination activity.

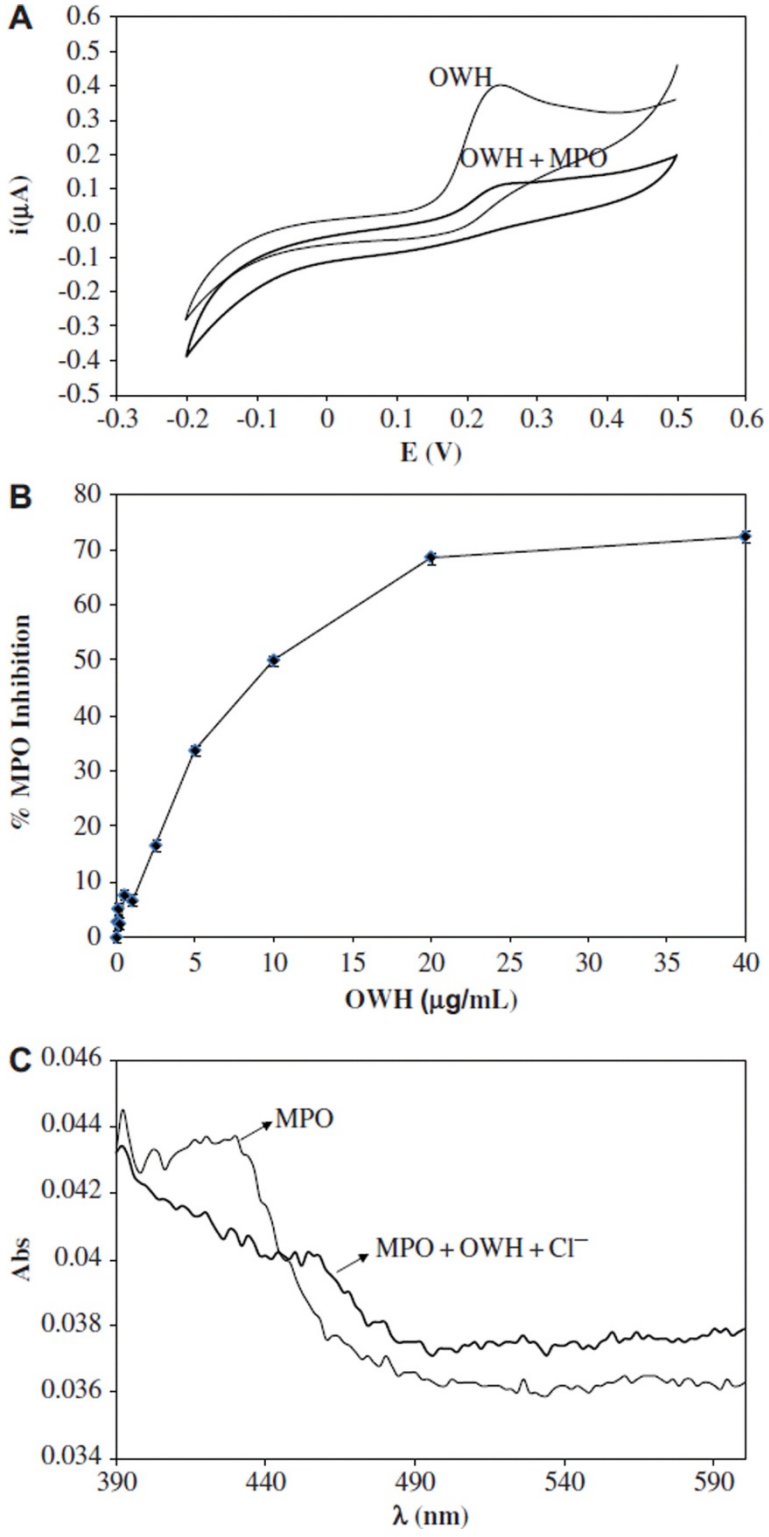


Figure 4.5. OWH myeloperoxidase inhibition. (A) Cyclic volta mmograms of 12 μg/mL of OWH in the absence (thin line) and presence (thick line) of MPO. 50 mM PBS pH 6.5, H2O2 100 μM and MPO 2 μM. Scan rate 5 mV/s. (B) Effect of OWH on MPO peroxidase activity. MPO 13 nM, guaiacol 10 mM and H_2O_2 100 μM in 50 mM PBS pH 6.6. Enzymatic reaction time 1 min. (C) Effect of OWH on the absorption spectrum of MPO. 50 mM PBS pH 6.5, 150 mM NaCl, H_2O_2 30 μM, MPO 1 μM and 7.5 μg/mL OWH. Spectra were recorded at 30 s.

4.3.3. Collagenase inhibition

Chronic wounds are known to contain elevated levels of matrix-degrading enzymes including proteases, glycosidases and several matrix metalloproteinases (MMPs) such as collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2, and MMP-9) or stromelysins (MMP-3, MMP-10 and MMP-11). Collagenases were the first MMPs to be discovered. This family of zinc-proteins is able to hydrolyze triple helical regions of collagen under physiological conditions. As in all MMPs, the collagenases contain a catalytic Zn^{2+} ion coordinated in their active center by three His residues. It is reported that some collagenase inhibitors bearing Zn^{2+} binding groups attack the zinc site in the catalytic domain of the enzyme [28].

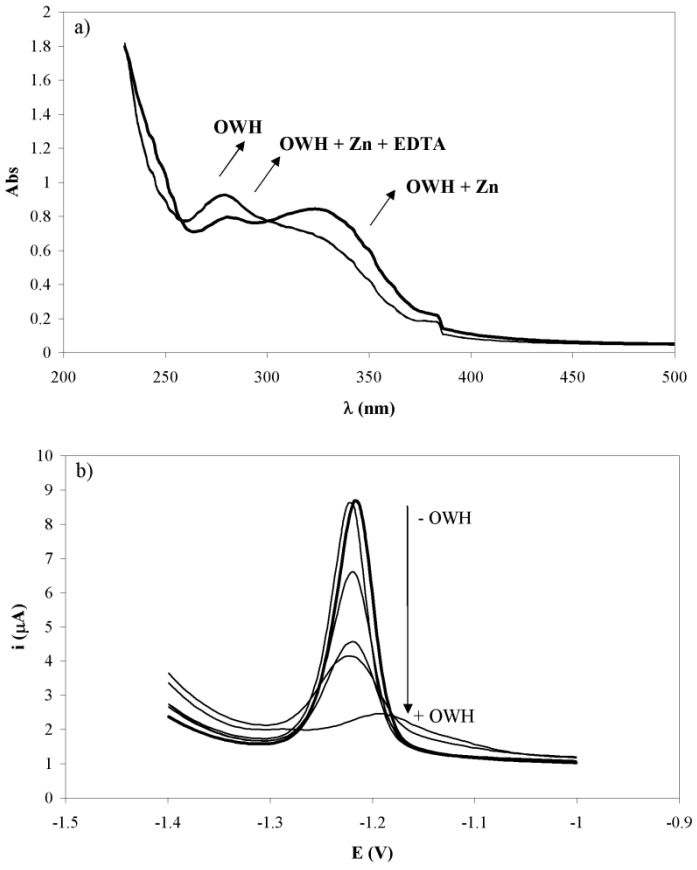


Figure 4.6. Zn^{2+} -binding capacity of OWH extract. (A) Absorption spectra for 100 μg/mL OWH after addition 1 mM Zn^{2+} and 1 mM EDTA; (B) DPASV of 25 μM Zn^{2+} in presence of different concentrations of OWH ranging from 0 to 15 μg/mL.

4.3. Results and discussion

The ability of the OWH extract to chelate Zn^{2+} ion was studied by both electrochemical and spectrophotometric assays. Polyphenols such as tannic acid have been previously reported to have Zn^{2+} -binding properties [29]. The UV–Vis spectra of the OWH extract (Figure 4.6A) shows a maximum absorbance in the region 250–300 nm. With the addition of Zn^{2+}, an additional band at 300–350 nm that is related to Zn^{2+} -polyphenol complex formation was found. This band disappears, and the spectrum returns to the initial one in the presence of EDTA, a stronger zinc chelator than the polyphenolic compounds. The interaction between Zn^{2+} and OWH extract was also confirmed by electrochemical measurements. As Figure 4.6B shows, the addition of OWH to Zn^{2+} solution results in a decrease in the peak current recorded by DPASV, which fact suggests the formation of a Zn^{2+} -polyphenol complex.

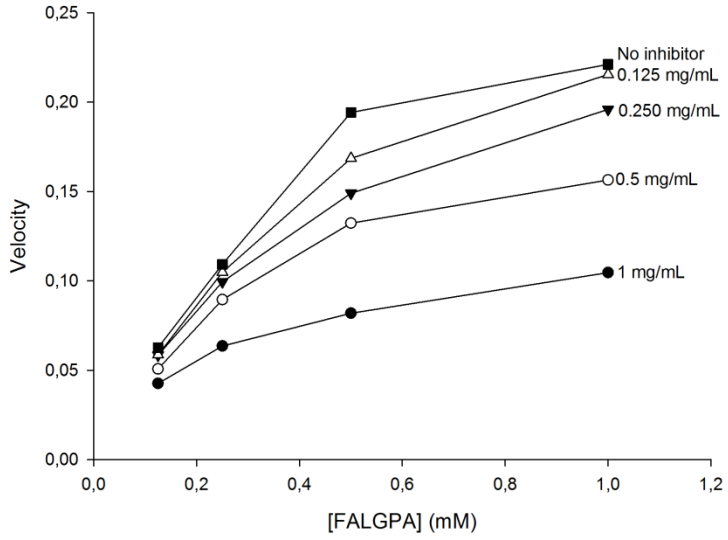


Figure 4.7. Collagenase inhibition by OWH. Assayed at different FALGPA concentrations, 0.2 µg/mL collagenase in 50 mM Tricine, 100 mM $CaCl_2$, 400 mM NaCl, pH 7.

The inhibitory effect of different concentrations of the OWH extract on FALGPA hydrolysis catalyzed by collagenase was evaluated. FALGPA is a short peptide, N-[3-(2-Furyl)acryloyl)]-Leu-Gly-Pro-Ala that can be used as synthetic substrate for collagenase activity [26]. The effect of OWH fraction on the hydrolysis of FALGPA by collagenase is shown in Figure 4.7. FALGPA hydrolysis is clearly suppressed in the presence of *H. virginiana* extract (IC_{50} = 75 ± 10 µg/mL). However, this inhibition was not reversed by the addition of Zn^{2+} as reported for other collagenase inhibitors [30]. This suggests that the major mechanism for inhibition of collagenase by the OWH extract was other than Zn^{2+} chelation. The inhibitory effect on

collagenase activity of green tea polyphenols—catechin (C) and epigallocatechin gallate (EGCG), has been recently reported [31]. Induced conformational changes in the collagenase secondary structure after treatment with the green tea polyphenols were assigned as the main reason for enzyme inhibition.

The direct-linear Eisenthal-Cornish-Bowden plot (Figure 4.8) describing the kinetics of Collagenase inhibition by extract revealed a mixed-type inhibition mechanism.

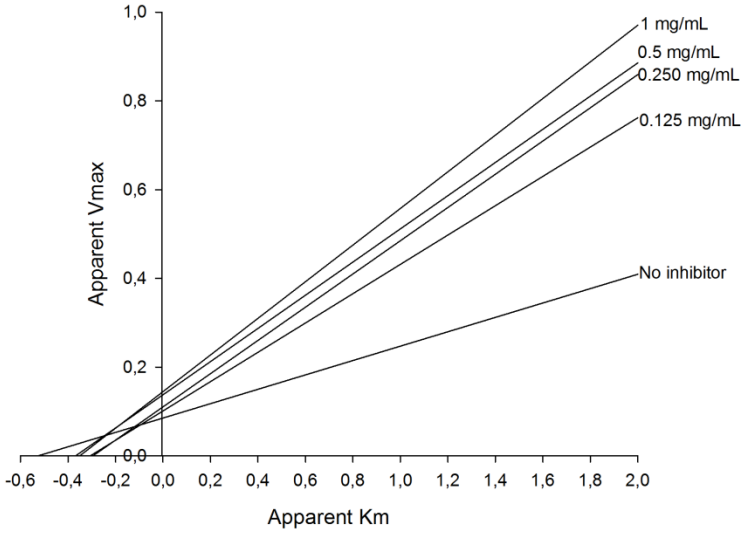


Figure 4.8. Direct-linear Eisenthal-Cornish-Bowden plots of the inhibition of ChC at various fixed concentrations of PA with FALGPA as a substrate. The enzyme (0.2 µg/mL) was incubated in 0.05 M Tricine buffer pH 7.5 (with 0.4 M NaCl and 0.01M $CaCl_2$, at 37 °C for 3 h) in presence of different PA concentrations. The reaction was initiated by addition of four different concentrations of FALGPA for each concentration of PA and the decrease of absorbance at 324 nm was monitored during 5 min to detect FALGPA hydrolysis products. The results represent mean values of three replicates.

The kinetic Collagenase-OWH dissociation constant (Kic) and Collagenase-FALGPA-OWH dissociation constant (Kiu) (Cornish-Bowden notation) were calculated by secondary plots of Kmapp/Vmaxapp vs [PA] and 1/Vapp vs [PA] and the Kic and Kiu values obtained were 6.1580 mM and 0.3904 mM respectively. These data suggest that the OWH bind both to Collagenase and the Collagense–FALGPA complex probably promoting conformational changes in the enzyme structure and inhibiting its activity [32]. The inhibitory effect on collagenase activity of green tea polyphenols—catechin (C) and epigallocatechin gallate (EGCG), has been recently reported [31]. Induced conformational changes in the collagenase secondary structure after treatment with the green tea polyphenols were assigned as the main reason for enzyme inhibition. Mixed-type inhibition may be considered as a consequence of the

presence of different phenolic compounds in *Hamamelis virginiana* extract exerting different inhibition effects [31-33].

4.4. Conclusions

Nowadays, the treatment of chronic wounds remains a major burden and drain on healthcare resources. Plant polyphenols from *H. virginiana* were found to have both a strong antioxidant activity and an inhibitory effect on MPO and collagenase – features potentially beneficial for the wound healing process. The extract used in this work showed a multifunctional antioxidant capacity. *H. virginiana* polyphenols were able to scavenge radical and non-radical species such as HOCl. Furthermore, their ability to act as peroxidase substrate for MPO results in inhibition of the chlorination activity of the enzyme, responsible for the HOCl production. Plant polyphenols also caused inhibition of the proteolytic activity of collagenase. As chronic ulcer fluids contain elevated levels of ROS and neutrophil-derived deleterious oxidative and proteolytic enzymes, the application of extract from *H. virginiana* seems to be a promising approach for use in the management of chronic wounds.

4.5. References

1. Diegelmann, R.F., *Excessive neutrophils characterize chronic pressure ulcers.* Wound Repair Regen, 2003. **11**(6): p. 490-5.
2. Rojkind, M., et al., *Role of hydrogen peroxide and oxidative stress in healing responses.* Cell Mol Life Sci, 2002. **59**(11): p. 1872-91.
3. Cawston, T.E. and A.J. Wilson, *Understanding the role of tissue degrading enzymes and their inhibitors in development and disease.* Best Pract Res Clin Rheumatol, 2006. **20**(5): p. 983-1002.
4. Trengove NJ, S.M., MacAuley S et al., *Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors.* Wound Repair and Regeneration, 1999. **7**: p. 442–452.
5. Agren, M.S., et al., *Collagenase in wound healing: effect of wound age and type.* J Invest Dermatol, 1992. **99**(6): p. 709-14.
6. Taylor, K.B., et al., *The mechanism of inhibition of collagenase by TIMP-1.* The Journal of Biological Chemistry, 1996. **271**(39): p. 23938-45.
7. Folkes, L.K., L.P. Candeias, and P. Wardman, *Kinetics and mechanisms of hypochlorous acid reactions.* Arch Biochem Biophys, 1995. **323**(1): p. 120-6.
8. Rice-Evans, C.A., N.J. Miller, and G. Paganga, *Structure-antioxidant activity relationships of flavonoids and phenolic acids.* Free Radic Biol Med, 1996. **20**(7): p. 933-56.
9. Mira, L., et al., *Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity.* Free Radic Res, 2002. **36**(11): p. 1199-208.
10. Pauff, J.M. and R. Hille, *Inhibition studies of bovine xanthine oxidase by luteolin, silibinin, quercetin, and curcumin.* J Nat Prod, 2009. **72**(4): p. 725-31.
11. Ding, Z.Q., et al., *Anti-Inflammatory Effects of Scopoletin and Underlying Mechanisms.* Pharmaceutical Biology, 2008. **46**(12): p. 854-860.
12. Geyid, A., et al., *Screening of some medicinal plants of Ethiopia for their anti-microbial properties and chemical profiles.* J Ethnopharmacol, 2005. **97**(3): p. 421-427.
13. Librandi, A.P.L., et al., *Effect of the extract of the tamarind (Tamarindus indica) fruit on the complement system: Studies in vitro and in hamsters submitted to a cholesterol-enriched diet.* Food and Chemical Toxicology, 2007. **45**(8): p. 1487-1495.
14. Kim, H., et al., *Enhanced wound healing by an epigallocatechin gallate-incorporated collagen sponge in diabetic mice.* Wound Repair and Regeneration, 2008. **16**(5): p. 714-720.
15. Amaral, S., et al., *Plant extracts with anti-inflammatory properties-A new approach for characterization of their bioactive compounds and establishment of structure-antioxidant activity relationships.* Bioorganic & Medicinal Chemistry, 2009. **17**(5): p. 1876-1883.
16. Deters, A., et al., *High molecular compounds (polysaccharides and proanthocyanidins) from Hamamelis virginiana bark: influence on human skin keratinocyte proliferation*

and differentiation and influence on irritated skin. Phytochemistry, 2001. **58**(6): p. 949-58.

17. Dauer, A., P. Metzner, and O. Schimmer, *Proanthocyanidins from the bark of Hamamelis virginiana exhibit antimutagenic properties against nitroaromatic compounds.* Planta Med, 1998. **64**(4): p. 324-7.
18. Tourino, S., et al., *Highly galloylated tannin fractions from witch hazel (Hamamelis virginiana) bark: electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells.* Chem Res Toxicol, 2008. **21**(3): p. 696-704.
19. Torres, J.L., et al., *Valorization of grape (Vitis vinifera) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content.* J. Agric. Food. Chem., 2002. **50**(26): p. 7548-55.
20. Torres, J. and A. Selga, *Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography.* Chromatographia, 2003. **57**(7): p. 441-445.
21. Touriño, S., et al., *Highly galloylated tannin fractions from witch hazel (Hamamelis virginiana) bark: electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells.* Chem. Res. Toxicol., 2008. **21**(3): p. 696-704.
22. Packer, L., *Flavonoids and Other Polyphenols*. 2001: Academic Press.
23. Dypbukt, J.M., et al., *A sensitive and selective assay for chloramine production by myeloperoxidase.* Free Radic Biol Med, 2005. **39**(11): p. 1468-77.
24. Gressier, B., et al., *SCAVENGING OF REACTIVE OXYGEN SPECIES BY LETOSTEINE, A MOLECULE WITH 2 BLOCKED -SH GROUPS - COMPARISON WITH FREE -SH DRUGS.* Pharmacy World & Science, 1995. **17**(3): p. 76-80.
25. Capeillere-Blandin, C., *Oxidation of guaiacol by myeloperoxidase: a two-electron-oxidized guaiacol transient species as a mediator of NADPH oxidation.* Biochemical Journal, 1998. **336 (Pt 2)**: p. 395-404.
26. Van Wart, H.E. and D.R. Steinbrink, *A continuous spectrophotometric assay for Clostridium histolyticum collagenase.* Analytical Biochemistry, 1981. **113**(2): p. 356-365.
27. Shiba, Y., et al., *Flavonoids as substrates and inhibitors of myeloperoxidase: Molecular actions of aglycone and metabolites.* Chemical Research in Toxicology, 2008. **21**(8): p. 1600-1609.
28. Rao, B.G., *Recent developments in the design of specific matrix metalloproteinase inhibitors aided by structural and computational studies.* Current Pharmaceutical Design, 2005. **11**(3): p. 295-322.
29. Cruz, B.H., et al., *Heavy metal binding by tannic acid: A voltammetric study.* Electroanalysis, 2000. **12**(14): p. 1130-1137.
30. Shiah, S.G., et al., *Inhibition of invasion and angiogenesis by zinc-chelating agent disulfiram.* Molecular Pharmacology, 2003. **64**(5): p. 1076-1084.
31. Madhan, B., et al., *Role of green tea polyphenols in the inhibition of collagenolytic activity by collagenase.* International Journal of Biological Macromolecules, 2007. **41**(1): p. 16-22.

32. Ganesan, K., et al., *Protective Effect of Withania somnifera and Cardiospermum halicacabum Extracts Against Collagenolytic Degradation of Collagen.* Applied Biochemistry and Biotechnology, 2011.

33. Kim, Y.-J., H. Uyama, and S. Kobayashi, *Inhibition effects of (+)-catechin–aldehyde polycondensates on proteinases causing proteolytic degradation of extracellular matrix.* Biochemical and Biophysical Research Communications, 2004. **320**(1): p. 256-261.

Chapter 5

A new approach to produce plant antioxidants-loaded chitosan for medical applications

Publications derived from this work:

Guillem Rocasalbas, Sonia Touriño, Josep Lluís Torres and Tzanko Tzanov. “A new approach to produce plant antioxidants-loaded chitosan for modulating proteolytic environment and bacterial growth” (Submitet)

5.1. Introduction

Chemical degradation methods for analysis of complex natural polymers such lignins and proanthocyanidins (PA) provide important compositional and structural information. These methods include among others acidolysis, thioacetolysis, hydrogenolysis, and the used in this paper thioacidolysis [1-3]. As an analytical method, thioacidolysis, alone or coupled with high-performance liquid chromatography (HPLC), has been used to characterize and quantify proanthocyanidins in plants and fruits [3]. The reaction, based on the lability of the interflavonoid linkages of PA in acid conditions and in presence of excess of nucleophile in the medium, results in the release of terminal subunits as flavan-3-ol monomers and extension subunits as electrophilic flavan-3-ol intermediates. The electrophilic intermediates are trapped by the nucleophile to give relatively stable derivates [4, 5]. Examples of suitable nucleophiles are: toluene-α-thiol (benzylmercaptan or phenylmethanethiol), from which the reaction was named “thioacidolysis”, phloroglucinol,[2] and cysteamine [6].

Recently, *Torres et al.* adapted this analytical method to synthesize new bio-based antioxidant compounds by depolymerization of plant-phenolic extracts in the presence of cysteamine and cysteine nucleophiles [3, 7-12]. The flavan-3-ol - thiol conjugates resulting from this reaction showed higher antiradical capacity [7, 8] and enhanced penetration through biological membranes [13] compared to their underivatized counterparts.

The depolymerization of proanthocyanidins in presence of macromolecular nucleophiles, such as chitosan, has never been used for permanent functionalization of biopolymers with phenolic moieties. Incorporation of natural antioxidant phenolic compounds from plant extracts onto chitosan have been reported by using tyrosinase [14] peroxidase [15] and laccase enzymes resulting in significant product oxidation [16]. The incorporation of natural antioxidant, e.g., gallic acid and (+)catechin, by free radical grafting [17, 18], or simply by adsorption onto chitosan resulted in high phenolics release and a rapid decrease of the antioxidant properties of the chitosan-phenol complex [19].

Here, for the first time, the analytical thioacidolysis reaction has been used to covalently graft phenolic moieties on a previously thiolated chitosan in order to generate bioactive materials for medical applications. Thiolated chitosan was selected as a suitable polysaccharide platform based on its biodegradability, biocompatibility, intrinsic antimicrobial activity, and lack of toxicity [20-22], in addition to its chemical structure providing the necessary to perform the reaction nucleophilic moieties. Furthermore, the presence of thiol groups significantly improves

the mucoadhesive and permeation properties of chitosan [23], whereas the in situ gelling properties of thiolated chitosan at physiological pH are particularly interesting for wound dressing materials [24]. The introduction of antioxidant phenolic compounds into the structure of chitosan will further broaden the range of potential medical applications of this biopolymer. The usefulness and efficiency of the developed material was evaluated against major factors governing the chronicity in wounds, e.g. matrix metalloproteinases (MMPs), myeloperoxidase (MPO), reactive oxygen species (ROS) and bacteria contamination.

The functionalization of thiolated chitosan was carried out with flavan-3-ols from witch hazel (*Hamamelis Virginiana*) extract. Witch hazel phenolics - proanthocyanidins and hydrolysable tannins - were able to protect red blood cells from free radical-induced hemolysis and inhibited the proliferation of tumoral SK-Mel 28 melanoma cells and HT29 and HCT116 human colon cancer cell lines. Moreover, this extract showed to be an effective protector against DNA damage triggered by hydroxyl radicals [11, 25], and exert an inhibitory effect over deleterious chronic wounds enzymes [26]. The method for permanent functionalization of chitosan developed in this paper is expected to combine the chemistry of amino, thiol and phenolic groups for obtaining biopolymer material with enhanced antioxidant capacity, antimicrobial effect and capability to modulate deleterious wound enzymes activity

5.2. Materials and methods

5.2.1 Materials

Chitosan from *Agaricus bisporus* (DA: 13%, MW: 50.7 KDa) was provided by KitoZyme (Herstal, Belgium). Witch hazel (*Hamamelis virginiana*) was purchased from Martin Bauer GmbH (Alveslohe, Germany). Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), 2-iminothiolane (TBA), gallic acid (GA), and chitosanase from S*treptomices griseus* were from Sigma-Aldrich (Spain). Collagenase from *Clostridium histolyticum* (1676 U/mg solid, 1 U hydrolyses 1.0 μmol of FALGPA per minute at pH 7.5 and 25 ºC in the presence of Ca^{2+}) and N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala (FALGPA) collagenase substrate from Sigma-Aldrich (Spain) were used. Myeloperoxidase (MPO) from human leukocytes (1550 U/mg solid: 1 U will produce an increase in absorbance at 470 nm of 1.0 per minute at pH 7.0 and 25 °C, calculated from the initial rate of reaction using guaiacol as a substrate) comes from Planta Natural Products (Austria). All other reagents used were of the highest grade commercially available from Sigma-Aldrich.

5.2.2 Extraction of proanthocyanidins from H. virginiana

Proanthocyanidins-rich fraction was extracted from witch hazel (*H. virginiana)* as previously reported in Chapter 4. The used extract for this experiment was AWH, rich in oligomeric proanthocyanidins and hydrolysable tannins (Table 5.1). Its degree of polymerization was about 6.23 estimated by thiolytic depolymerization and high performance liquid chromatography (HPLC) using appropriate molecular standards (Chapter 4).

Table 5.1. Polyphenolic composition of AWH witch hazel extract

Phenolic compounds	**Percentage**
Catechins (catechin and epicatechin)	17.8
Gallocatechins (gallocatechin, epigallocatechin)	14.5
Catechingallates (epigallocatechin gallate, epicatechin gallate)	25.5
Hydrolizable tannins	42.2
Total phenol content (mg GAE/g fraction)	261±10

5.2.3. Thiolation of chitosan

The incorporation of thiol groups in chitosan was carried out following the procedure developed by Bernkopf-Schnürch et al. [27]. Briefly, chitosan was dissolved in HCl (1% v/v). Different amounts of 2-iminothiolane HCl (Traut's reagent) (Table 5.2) were added and the pH was

adjusted to 6 with 5 M NaOH.The reaction mixture was stirred for 24 h at room temperature. The resulting thiolated polymer was dialyzed against different aqueous media in the following order: i) 5 mM HCl, ii) twice against 5 mM HCl containing 1% NaCl, iii) 5 mM HCl, and finally iv) 1 mM HCl. Thereafter, the chitosan thiol derivatives, as specified in Table 2, were freeze-dried. The degree of thiol modification of chitosan was estimated spectrophotometrically using Ellman's reagent [28]. The samples were dissolved in mQ water at final concentration of 1 mg/mL.

Aliquots (250 μL) of the solutions, 250 μL 0.5 M phosphate buffer pH 8.0, and 500 μL Ellman's reagent (dissolved in 10 mL 0.5 M phosphate buffer pH 8.0) were reacted. The reaction was allowed to proceed for 2 h at room temperature. Thereafter, the polymer was removed by centrifugation, and 300 μL of the supernatant were transferred to a microtitration plate. The absorbance was immediately measured at 450 nm with a microplate reader Infinite M200, Tecan, Austria. Non-thiolated chitosan was used as a blank. The amount of thiol moieties was calculated from a standard curve obtained with cysteine–HCl solutions.

Table 5.2. Thiolated chitosan samples

Thiolated chitosan		
Sample code	**Reaction ratio Chitosan:Traut's**	**μmols free SH/g polymer**
C	1:00	0.00
C-TBA.1	10:01	21.10 ± 0.32
C-TBA.2	2:01	140.00 ± 3.48

5.2.4. Incorporation of flavan-3-ol moieties from Hamamelis virginiana *onto chitosan via thiolysis*

A modified thiolysis procedure adopted from Selga and Torres [6] was used to obtain phenolics-loaded chitosan. Samples of chitosan with different degree of thiolation (table 2) were dissolved in MeOH:H_2O:HCl (43:55:2), then the *H. virginiana* extract was added at final concentration of 0.3 mg/mL. The reaction was carried at 80 ºC for 15 min. After cooling, the solutions were dialyzed several times at room temperature against distilled water and then freeze-dried. The controls were obtained submitting the same samples to thiolysis reaction at room temperature (RT). The samples are specified in Table 5.3.

Table 4.3. Samples of thiolated chitosan covalently loaded with phenolic moieties.

Sample code	Initial conditions		
	Initial sample	Reaction temperature	PA (mg/mL)
C-PA	C	80 ºC	0.3
C-TBA.1-PA	C-TBA.1	80ºC	0.3
C-TBA.2-PA	C-TBA.2	80ºC	0.3
C-PA-RT	C	RT	0.3
C-TBA.1-PA-RT	C-TBA.1	RT	0.3
C-TBA.2-PA-RT	C-TBA.2	RT	0.3

5.2.4.1. Determination of the total phenolic content

The total phenolic content of the samples dissolved in mQ water was determined spectrophotometrically (Infinite M200, Tecan, Austria) at 280 nm and expressed in gallic acid equivalents (GAE) using the equation obtained from the calibration curve performed with gallic acid in the same conditions.

5.2.4.2. FTIR characterization

Infrared spectra of the samples were collected over the 3500 - 800 cm^{-1} range using Perkin-Elmer Spectrum 100 (Perkin-Elmer, Massachusetts, USA) spectrophotometer equipped with universal Attenuated Total Reflectance (ATR) sampling accessory, performing 50 scans for each spectrum.

5.2.4.3. NMR analysis

^{1}H NMR analyses were carried out on a Bruker Avance III 500 MHz spectrometer (600MHz) at 298 K.

5.2.4.4. HPLC characterization

Previous to the analysis the C-TBA.2-PA sample was incubated in acetate buffer (0.1 M, pH 6) containing 5 U of chitosanase, at 37 ºC, under continuous shaking. After 24 h of incubation another 5 U of enzyme were added and the reaction mixture was further incubated for 24 h. Then, it was centrifuged using Vivaspin 3000 MWCO (Sartorius Stedim Biotech S.A.) in order to separate the enzyme and non-hydrolyzed sample from the hydrolyzed oligomers. The resulting solution was lyophilized and re-dissolved in 0.005 M H_2SO_4 in mQ water to obtain solutions with defined concentration prior to the HPLC analysis. The HPLC analysis of the products of the enzymatic degradation of C-TBA.2-PA was carried out with HPLC-Agilent Technologies 1200, equipped with diode array detector (DAD, Agilent Technologies 1200) and refractive index detector (RID, Agilent Technologies 1200) coupled to a Bio-Rad aminex HPX-

87 H column using constant flow rate of 0.6 mL/min with isocratic elution of 0.005 M H_2SO_4 as mobile phase, at 100 µL injection volume.

5.2.5. In vitro *tests to evaluated biological activities*

5.2.5.1. Radical scavenging activity

The radical scavenging activity was determined spectrophotometrically measuring the decrease in absorbance of 1,1-diphenyl-2-picrylhydrazyl radical (DDPH) at 515 nm [29]. To measure the DPPH scavenging effect, chitosan and functionalized chitosans were dissolved in mQ H_2O, then 200 µL of the polymer solution were added to 1400 µL $6x10^{-5}$ M DPPH solution in MeOH, and incubated at room temperature in the dark for 15 min. All experiments were carried out in triplicate and the results are expressed as a percentage of DPPH inhibition.

5.2.5.2. Antimicrobial test

The minimum inhibitory concentration (MIC) of chitosan and/or modified chitosan was determined by a turbidimetric method [30] against *Pseudomonas Aeruginosa* and *Staphylococcus aureus*. The positive control was obtained with doxycycline. The samples were studied for visible signs of growth or turbidity. The lowest concentration of chitosan and/or modified chitosan that inhibited the growth of bacteria was considered as the minimum inhibitory concentration (MIC).

5.2.5.3. MPO inhibition

The inhibitory effect of the blends over MPO activity was measured using guaiacol as a substrate [31]. Samples were incubated with 20 µL of MPO 0.325 µM (0.60 U) and 30 µL of guaiacol (167 mM) buffered with 450 µL PBS pH 7.4 at 37 °C. After 3h incubation time 200 µL of the solution were placed in a 96-well microplate and the reaction was started by adding 22 µL of 1 mM H_2O_2. The change in absorbance at 470 nm was monitored during 5 cycles of 15 sec (75 sec.) using microplate reader Infinite M200 (Tecan, Austria). All measurements were carried out in triplicate and the activity was determined by the rate of absorbance increase per min and expressed as a percentage of enzyme inhibition compared to the control (MPO reaction mixture without sample).

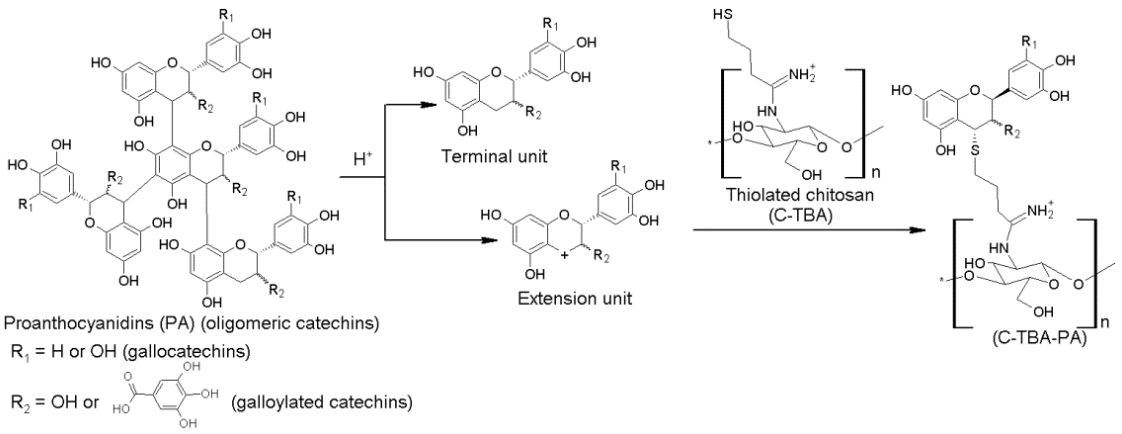
5.2.5.4. Collagenase inhibition

The collagenase activity was measured using FALGPA substrate [32]. The Collagenase was incubated in presence of samples for 3h of incubation. The hydrolysis of FALGPA with collagenase 0.2 µg/mL in 50 mM Tricine, containing 100 mM $CaCl_2$ and 400 mM NaCl, pH 7.4 was monitored spectrophotometrically at 345 nm during 5 min, immediately after mixing the substrate with the enzyme. All measurements were carried out in triplicate. The results were expressed as a percentage of enzyme inhibition compared to the control (collagenase raction mixture without sample).

5.3. Results and discussion

5.3.1 Functionalization of thiolated chitosan with flavan- 3-ol via thiolysis reaction.

Reacting thiolated chitosan (C-TBA) with proanthocyanidins of *Hamamelis virginiana* in acidic condition was expected to yield chitosan covalently functionalized with phenolic moieties (C-TBA-PA). Under acidic conditions, oligomeric proanthocyanidins depolymerize and the electrophilic intermediates can be trapped by a nucleophile through the C4 position in flavan-3-ol (Scheme 5.1). Both amino groups of chitosan and the amidine substructure of thio-buthyl-amidine moiety of thiolated chitosan (C-TBA) are protonated in acidic medium, thus, only the thiol groups can act as nucleophiles reacting with the electrophilic PA intermediates via thioether bonds. Therefore, the thiolysis reaction provides selective functionalization of thiolated chitosan through thioether linkages in a single step approach.



Scheme 5.1. Scheme of the thiolysis reaction of proanthocyanidins (PA) in presence of thiolated chitosan

Unmodified chitosan, C-TBA.1 and C-TBA.2 sample were reacted with the same amount of PA extract (0.3 mg/mL) at 80 ºC for 15 min. The results showed that the amount of grafted polyphenols via thiolysis on the thiolated chitosan increased depending on the degree of thiolation of the biopolymer (Figure 5.1). Polyphenols were also detected on the non thiolated chitosan (C-PA) possibly due to electrostatic interactions with the positively charged amino group of chitosan, additional hydrogen-bonds between phenolic groups and chitosan network, and hydrophobic interaction [19].

Selga et al. [33] observed that the increase of the temperature boosts thedepolymerization reaction. Accordingly, we carried out the thiolysis both at room temperature (RT) and at 80 ºC. When the reaction was carried out at RT the amount of phenolics was similar on all chitosan samples regardless of their thiol content, while at 80 ºC a nearly 3-fold increase of the amount of polyphenols on the thiolated chitosan was observed (Figure 5.1). The results showed that the interaction between flavan-3-ol moieties and thiolated chitosan increased with the increase of the thiolation degree at temperatures favoring the PA depolymerization. The sample with highest degree of functionalization C-TBA.2-PA was selected for further experiments.

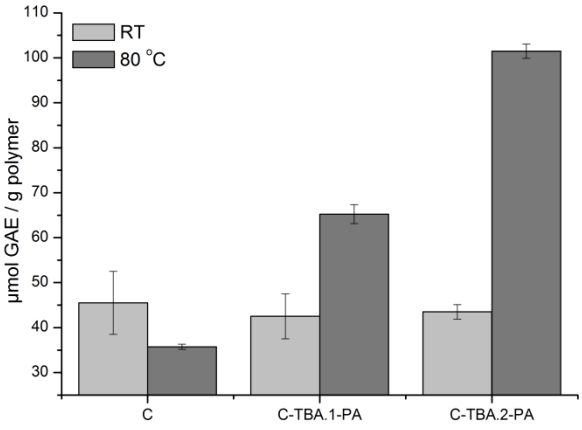


Figure 5.1. Amount of phenolics incorporated on thiolated chitosan as a function of the degree of thiolation and the reaction temperature.

The band at 1630 cm^{-1} characteristic for C=O stretching of carboxylic acids and amides in the FTIR spectra of chitosan increased in the case of C-TBA.2 and C-TBA.2-PA due to the imino group (C=N), which absorption peak is close to the carbonyl (C=O) stretching region [34] (Figure 5.2). At the same time the band at 1203 cm^{-1} assigned to C-N stretching of amines increased after functionalization of chitosan, pointing out the formation of new C-N bounds. In the chitosan spectra a band at 1593 cm^{-1} was observed corresponding to NH_3^+ salts that disappeared after thiolation. These observations confirm the incorporation of Traut's reagent though the amino groups of chitosan. In addition the band at 1540 cm^{-1} in C-TBA spectra shifted to 1522 cm^{-1} in C-TBA-PA spectra. This band corresponds to N-H of secondary amides suggesting non-covalent interactions between secondary amides of chitosan and PA. These expected non-covalent interactions were also observed in Figure 4.1 when the thiolysis reaction was conducted at RT. The absorption of the thiol group is the only peak that can be considered to determine the thioether linkage. Unfortunately, the equivalent C-S and C-S-H stretching

vibrations tend to give rise to a very weak absorption in the region of 2600 - 2550 cm^{-1} for S-H stretching and 715 – 630 cm^{-1} for C-S stretching [34]. Consequently, the information provided by the FTIR analysis revealed not to be suitable for identification of the thioether bond.

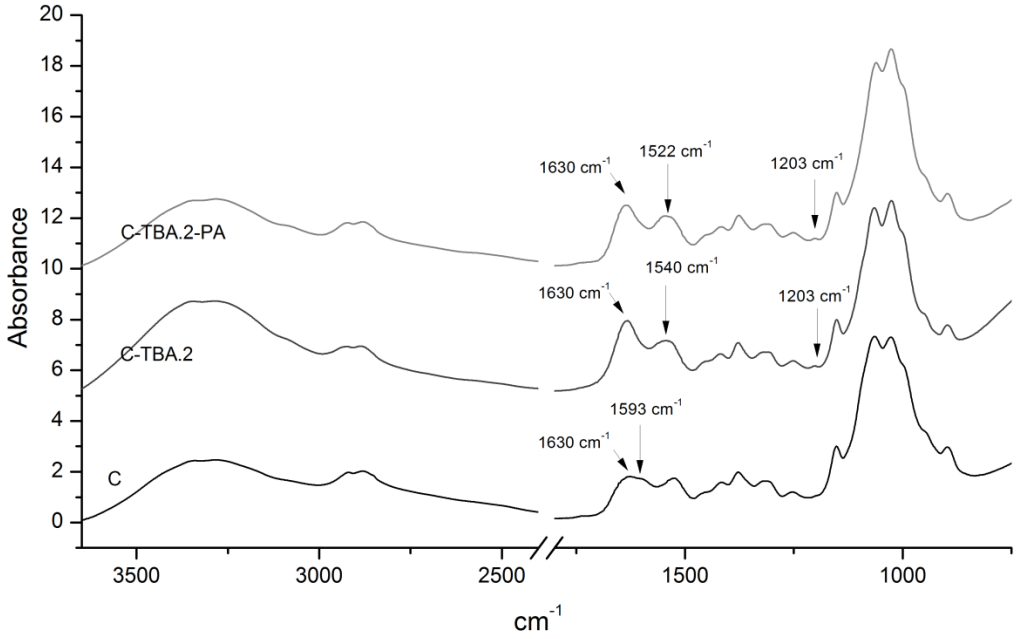


Figure 5.2. Normalized FTIR spectra of freeze-dried C, C/-TBA.2 and C-TBA.2-PA samples.

The ^{1}H NMR spectra of non-functionalized chitosan (Figure 5.3A) shows characteristic peaks of the glucosoamine residues at 4.65 ppm for the anomeric proton on C-1, and at 2.91 ppm for the proton on C-2. The peak for the methyl protons of the N-acetylglucosamine residues appears at 2.07 ppm, and the peaks between 3.4 and 4.1 ppm correspond to protons on C-3, C-4, C-5, and C-6. The C-TGA.2 showed new peaks at 2.77 ppm, 2.57 ppm, 2.45 ppm, and 1.97 ppm (Figure 5.3B) assigned to the thio-buthyl-amidine, thereby confirming the thiolation of chitosan. The peaks at 7.02 ppm and 7.12 ppm belong to phenyl proton signals [8] confirming the presence of phenolic moieties in C-TBA.2-PA structure (Figure 5.3C). The resonance signal at 2.89 ppm of the adjacent to thiol group protons for C-TBA.2 was rather weak due to the low thiol content [35]. Moreover, it is partially overlapped by the signal of the C-2 protons. For the C-TBA.2-PA this signal is completely overlapped by C-2 signal due to a shift to a lower resonance field. The thioether spectrum is distinguishable from the thiol spectrum by the shift to a lower field of the protons adjacent to the sulfur atom.

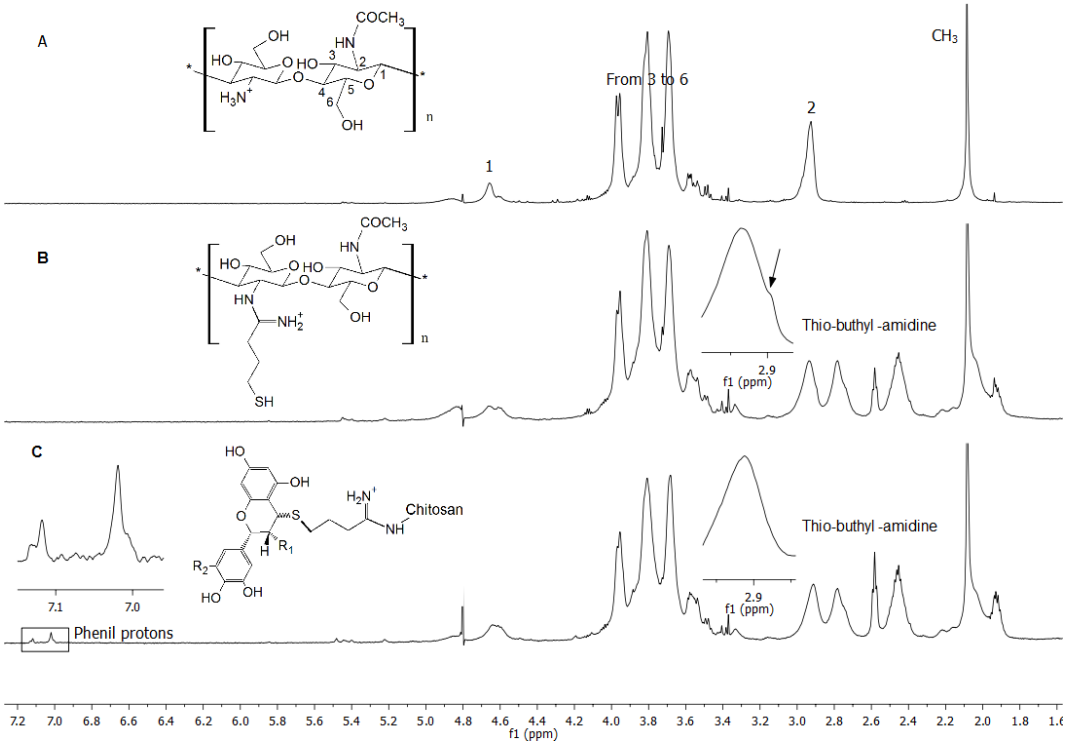


Figure 5. 3. ^{1}H NMR spectra of (A) chitosan, (B) C-TBA.2 and (C) C-TBA.2-PA

The HPLC analysis of C-TBA.2-PA was preceded by chitosanase hydrolysis of the samples. The purpose of this hydrolysis was to obtain covalently functionalized with phenolics carbohydrate oligomers to be easily analyzed by HPLC equipped with refractive index detector (RID) and diode array detector (DAD) at 280 nm. The soluble sugars from the enzymatically-hydrolyzed chitosan can be separated using a specific ion exchange HPLC Aminex HPX-87H and detected by RID, while the absorption band of the phenolic compounds at 280 nm is easily identifiable with the UV detector. Using a double detection, e.g. UV at 280 nm followed by RID, allowed to discriminate among: i) unmodified sugars not detectable at 280 nm, ii) unreacted PA not detectable in RID, and iii) sugars functionalized with phenolic moieties, visible both in RID and at 280 nm. The chromatograms obtained using RID and DAD 280 nm showed common peaks detected at 16 min and 21 min (Figure 5.4A and 5.4B respectively). These two peaks, observed with both detectors, correspond to hydrolyzed sugars covalently grafted with flavonols through thiolysis reaction. The other peaks observed only at 280 nm corresponds to unbound phenolic moieties meanwhile the peaks detected only in RID correspond to unmodified soluble sugars from hydrolysis of chitosan. These evidences together with the ^{1}H NMR results support the hypothesis that flavanols are covalently grafted with the C-TBA through thioether bond.

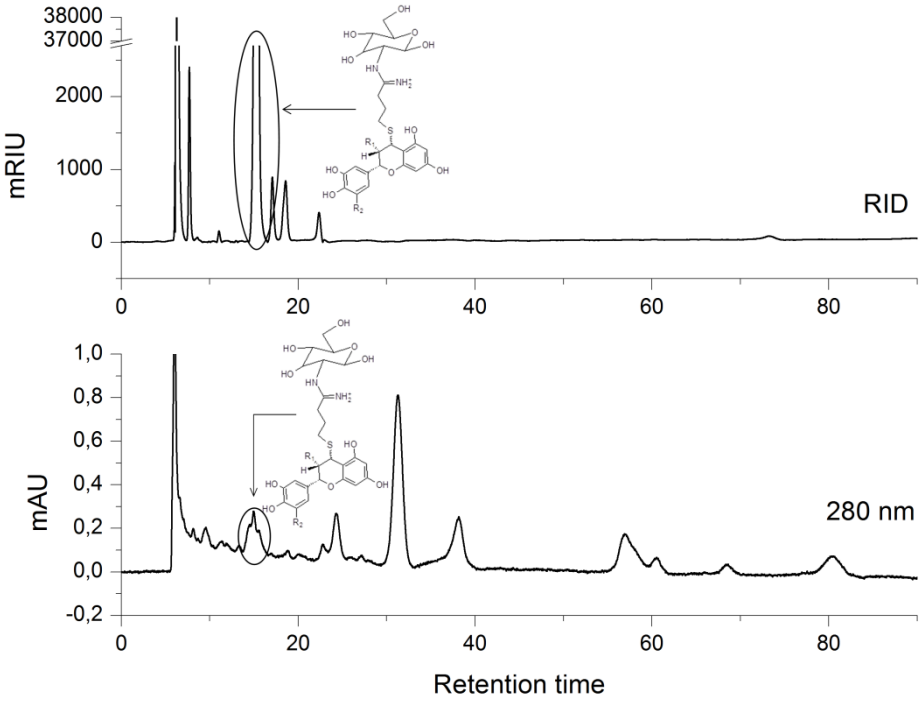


Figure 5.4. RID and DAD chromatograms of hydrolysis products of functionalized C-TBA.2-PA.

5.3.2. Antioxidant, antimicrobial and enzymatic inhibitory properties of phenolics-modified chitosan

DPPH assay revealed an improvement of the radical scavenger activity of chitosan after functionalization via thiolysis (Figure 5.5). The presence of thiol and phenolic moieties enhanced synergistically the antioxidant activity of functionalized chitosan compared to the unmodified chitosan and thiolated chitosan. The antioxidant capacity of the material should increase with its hydrogen-donating ability. Thiols are known to possess strong hydrogen-donating character, and as a consequence, the DPPH scavenger activity of C-TBA.2 increased five-fold compared to the unmodified chitosan. On the other hand, plant phenolics comprise one of the major groups of compounds acting as primary antioxidants or free radical scavengers [36] based on the ability of their phenolic moieties to donate hydrogen. The conjugation of flavan-3-ol moieties and thiol groups results in a chitosan derivative (C-TBA.2-PA) with nearly twenty-five fold higher antioxidant capacity compared to the unmodified chitosan, and five-fold higher in respect to the thiolated chitosan (sample C-TBA.2). In addition Torres et al. [7] observed that the new thioether bond in C4 position (Scheme 5.1) of the cysteinyl-flavan-3-ol conjugates enhanced their DPPH scavenger activity when compared with underivatized flavonoids. Thus, the functionalization of chitosan with both thiol and phenolic moieties exerts a synergistic effect

enhancing the antioxidant capacity of the biopolymer by an increase of the number of hydrogen-donor groups, and the presence of thioether bond in the flavan-3-ol derivatives.

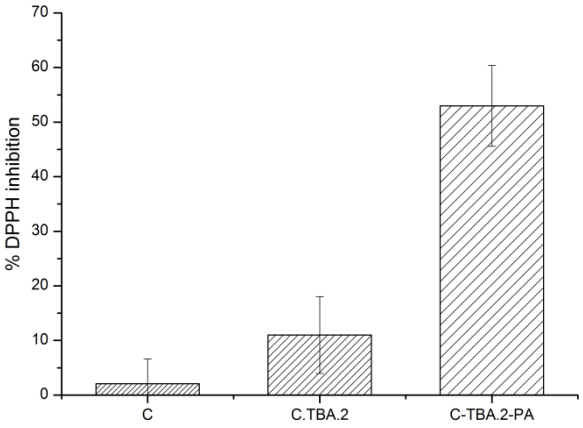


Figure 5.5. DPPH radical scavenging activity of chitosan (C), thiolated chitosan (C-TBA-2), and phenolics-functionalized chitosan (C-TBA-PA).

The antimicrobial effect of unmodified chitosan was much greater for Gram positive (*S. aureus*) than for Gram negative (*P. aeruginosa*) bacteria. The phenolics-chitosan conjugate improved the antimicrobial behavior of chitosan against *P. aeruginosa*, while decreased its antimicrobial capacity against *S. aureus*. The incorporation of polyphenols, in addition to the thiol modification lowered the antimicrobial effect in the case of *S. aureus*, probably due to the reduced availability of free amino groups in the sample. In the case of *P. aeruginosa* an enhancement of antimicrobial effect was observed after thiolation and phenolics incorporation (Table 5.4).

The most widely accepted mechanism for bacteria inhibition by chitosan is through the interaction between its cationic amino groups and negatively charged microbial cell membranes, resulting in the alteration of the membrane wall permeability. This mechanism has been reported to be common for both strains but exhibit a stronger effect on the cell membrane of *S. aureus* [37]. The decrease of antimicrobial activity against *Staphylococcus aureus* was due to a decrease of the amount of free amino groups, while the increase of the antibacterial activity against *Pseudomonas aeruginosa*, could be due to the presence of phenolic moieties [38]. Higher functionalization degree means lower availability of free amino groups in chitosan structure, reflected negatively in its antibacterial capacity against *S. aureus.* The tolerance of bacteria for phenol compounds depends on the bacteria species and the molecular structure of the phenolics. The present extract is rich in epigallocatechins gallate (Table 5.1) that have been

reported to possess antimicrobial activity against *P. aeruginosa* [39] through damaging the cell membrane.

Table 5.4. Inhibitory effect of chitosan-phenolics samples after 24 h incubation with *S. aureus* and *P. aeruginosa.*

	(MIC (mg/mL)	
Sample name	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
C	<1	>500
C-TBA.2	8	250
C-TBA.2-PA	8	125

In this study, thiolated chitosan (C-TBA.2) showed improved inhibitory activity over collagenase and MPO compared to the unmodified polymer (Fig. 6). Thiolated polymers have been already reported to possess inhibitory effect against collagenase and MPO activity as a function of the degree of thiolation [40]. Nevertheless, the inhibition of collagenase and MPO in the presence of thiolated chitosan functionalized with PA (C-TBA.2-PA) further increased by 22% and 32% respectively, pointing out the effectiveness of PA incorporation. Our previous studies showed a dose-dependent collagenase and MPO inhibition by *H. virginiana* extracts [26]. Translated to the wound environment, this combined MPO and collagenase inhibition would lead to the attenuation of enzymes activity and consequently allow the healing to progress.

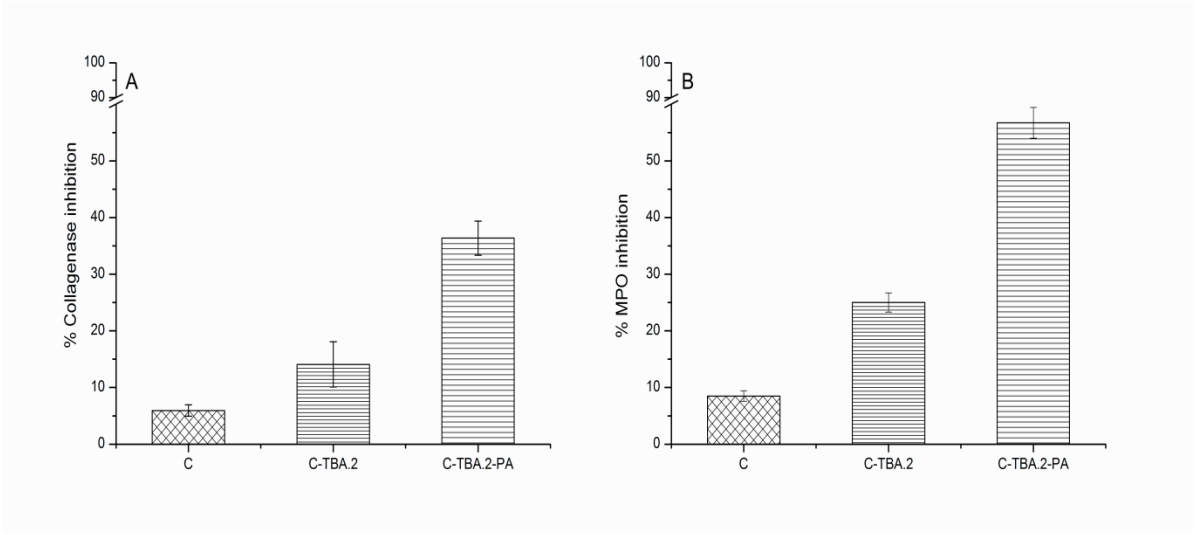


Figure 5.6. Effect of chitosan functionalization on major chronic wound enzyme activities A) Collagenase and B) MPO. All results are mean values of three replicates ± standard deviation.

5.5. Conclusion

A new method for covalent grafting of plant-derived antioxidant polyphenols on high molecular weight substrates, e.g. chitosan using thioacidolysis reaction was developed. The generated products combine multiple functional groups, such as amino, thiol, imino and phenolic moieties. The new compounds described here result from the breakdown of oligomeric proanthocyanidins in the presence of thiolated chitosan. The new chitosan conjugate (C-TBA.2-PA) showed increased antioxidant activity and inhibitory capacity over both collagenase and MPO enzymatic activities in comparison with the non-functionalized chitosan (C), and thiolated chitosan (C-TBA.2). Furthermore, the new conjugate showed increased antimicrobial efficiency against the Gram negative *Pseudomonas aeruginosa.* Both features, strong antioxidant activity and an inhibitory effect on *P. aeruginosa*, are potentially beneficial for wound healing processes. Therefore thioacidolysis seems to be a promising approach to obtain biopolymeric matrices with enhanced functional properties for wound treatment applications.

5.5. References

1. Brunow, G., *Methods to Reveal the Structure of Lignin*, in *Biopolymers Online*. 2005, Wiley-VCH Verlag GmbH & Co. KGaA.

2. Matthews, S., et al., *Method for Estimation of Proanthocyanidins Based on Their Acid Depolymerization in the Presence of Nucleophiles.* J. Agric. Food Chem., 1997. **45**(4): p. 1195-1201.

3. Torres, J. and A. Selga, *Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography.* Chromatographia, 2003. **57**(7): p. 441-445.

4. Matthews, S., et al., *Method for Estimation of Proanthocyanidins Based on Their Acid Depolymerization in the Presence of Nucleophiles.* J. Agric. Food Chem., 1997. **45**(4): p. 1195-1201.

5. Thompson, R.S., et al., *Plant proanthocyanidins. Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins.* J. Chem. Soc., Perkin Trans. 1, 1972: p. 1387-1399.

6. Selga, A. and J.L. Torres, *Efficient preparation of catechin thio conjugates by one step extraction/depolymerization of pine (Pinus pinaster) bark procyanidins.* J. Agric. Food Chem., 2005. **53**(20): p. 7760-5.

7. Torres, J.L. and R. Bobet, *New Flavanol Derivatives from Grape (Vitis vinifera) Byproducts. Antioxidant Aminoethylthio−Flavan-3-ol Conjugates from a Polymeric Waste Fraction Used as a Source of Flavanols.* J. Agric. Food Chem., 2001. **49**(10): p. 4627-4634.

8. Torres, J.L., et al., *Cysteinyl-flavan-3-ol conjugates from grape procyanidins. Antioxidant and antiproliferative properties.* Bioorg. Med. Chem., 2002. **10**(8): p. 2497-509.

9. Torres, J.L., C. Lozano, and P. Maher, *Conjugation of catechins with cysteine generates antioxidant compounds with enhanced neuroprotective activity.* Phytochemistry, 2005. **66**(17): p. 2032-7.

10. Torres, J.L., et al., *Valorization of grape (Vitis vinifera) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content.* J. Agric. Food Chem., 2002. **50**(26): p. 7548-55.

11. Touriño, S., et al., *Highly galloylated tannin fractions from witch hazel (Hamamelis virginiana) bark: electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells.* Chem. Res. Toxicol., 2008. **21**(3): p. 696-704.

12. Touriño, S., et al., *Procyanidin fractions from pine (Pinus pinaster) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells.* J. Agric. Food Chem., 2005. **53**(12): p. 4728-35.

13. Alonso, C., et al., *Percutaneous absorption of flavan-3-ol conjugates from plant procyanidins.* Drugs. Exp. Clin. Res., 2004. **30**(1): p. 1-10.

14. Muzzarelli, R.A.A., et al., *Tyrosinase-mediated quinone tanning of chitinous materials.* Carbohydr. Polym., 1994. **24**(4): p. 295-300.

15. Pasanphan, W., G.R. Buettner, and S. Chirachanchai, *Chitosan gallate as a novel potential polysaccharide antioxidant: an EPR study.* Carbohydr. Res., 2010. **345**(1): p. 132-140.

16. Chen, T., et al., *Enzymatic grafting of hexyloxyphenol onto chitosan to alter surface and rheological properties.* Biotechnol. Bioeng., 2000. **70**(5): p. 564-73.

17. Curcio, M., et al., *Covalent insertion of antioxidant molecules on chitosan by a free radical grafting procedure.* J. Agric. Food Chem., 2009. **57**(13): p. 5933-8.

18. Cho, Y.-S., et al., *Preparation, characterization, and antioxidant properties of gallic acid-grafted-chitosans.* Carbohydr. Polym., 2011. **83**(4): p. 1617-1622.

19. Popa, M.-I., et al., *Study of the interactions between polyphenolic compounds and chitosan.* React. Funct. Polym., 2000. **45**(1): p. 35-43.

20. Jayakumar, R., et al., *Biomaterials based on chitin and chitosan in wound dressing applications.* Biotechnol. Adv., 2011. **29**(3): p. 322-37.

21. Alves, N.M. and J.F. Mano, *Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications.* Int. J. Biol. Macromol., 2008. **43**(5): p. 401-14.

22. Jayakumar, R., et al., *Novel Chitin and Chitosan Materials in Wound Dressing*, in *Biomedical Engineering, Trends in Materials Science*, A.N. Laskovski, Editor. 2011.

23. Bernkop-Schnurch, A., M. Hornof, and D. Guggi, *Thiolated chitosans.* Eur. J. Pharm. Biopharm., 2004. **57**(1): p. 9-17.

24. Sakloetsakun, D., J.M. Hombach, and A. Bernkop-Schnurch, *In situ gelling properties of chitosan-thioglycolic acid conjugate in the presence of oxidizing agents.* Biomaterials, 2009. **30**(31): p. 6151-7.

25. Lizárraga, D., et al., *Witch Hazel (Hamamelis virginiana) Fractions and the Importance of Gallate Moieties□Electron Transfer Capacities in Their Antitumoral Properties.* J. Agric. Food Chem., 2008. **56**(24): p. 11675-11682.

26. Díaz-González, M., et al., *Inhibition of deleterious chronic wound enzymes with plant polyphenols.* Biocat. Biotrans., 2012. **30**(1): p. 102-110.

27. Bernkop-Schnurch, A., M. Hornof, and T. Zoidl, *Thiolated polymers--thiomers: synthesis and in vitro evaluation of chitosan-2-iminothiolane conjugates.* Int. J. Pharm., 2003. **260**(2): p. 229-37.

28. Bravo-Osuna, I., et al., *Characterization of chitosan thiolation and application to thiol quantification onto nanoparticle surface.* Int. J. Pharm., 2007. **340**(1-2): p. 173-81.

29. Brand-Williams, W., M.E. Cuvelier, and C. Berset, *Use of a free radical method to evaluate antioxidant activity.* LWT - Food. Sci. Technol., 1995. **28**(1): p. 25-30.

30. Qi, L., et al., *Preparation and antibacterial activity of chitosan nanoparticles.* Carbohydr. Res., 2004. **339**(16): p. 2693-700.

31. Capeillere-Blandin, C., *Oxidation of guaiacol by myeloperoxidase: a two-electron-oxidized guaiacol transient species as a mediator of NADPH oxidation.* Biochemical Journal, 1998. **336 (Pt 2)**: p. 395-404.

32. Van Wart, H.E. and D.R. Steinbrink, *A continuous spectrophotometric assay for Clostridium histolyticum collagenase.* Analytical Biochemistry, 1981. **113**(2): p. 356-365.

33. Selga, A., et al., *Efficient one pot extraction and depolymerization of grape (Vitis vinifera) pomace procyanidins for the preparation of antioxidant thio-conjugates.* J. Agric. Food Chem., 2004. **52**(3): p. 467-73.

34. Coates, J., *Interpretation of Infrared Spectra, A Practical Approach.* Encyclopedia of Analytical Chemistry.

35. Zhang, H., A. Qadeer, and W. Chen, *In Situ Gelable Interpenetrating Double Network Hydrogel Formulated from Binary Components: Thiolated Chitosan and Oxidized Dextran.* Biomacromolecules, 2011. **12**(5): p. 1428-1437.

36. Sánchez-Moreno, C., J.A. Larrauri, and F. Saura-Calixto, *A procedure to measure the antiradical efficiency of polyphenols.* J. Sci. Food. Agric., 1998. **76**(2): p. 270-276.

37. Tao, Y., L.-H. Qian, and J. Xie, *Effect of chitosan on membrane permeability and cell morphology of Pseudomonas aeruginosa and Staphyloccocus aureus.* Carbohydr. Polym., 2011. **86**(2): p. 969-974.

38. De Bruyne, T., et al., *Biological Evaluation of Proanthocyanidin Dimers and Related Polyphenols.* J. Nat. Prod., 1999. **62**(7): p. 954-958.

39. Xie, W., P. Xu, and Q. Liu, *Antioxidant activity of water-soluble chitosan derivatives.* Bioorg. Med. Chem. Lett., 2001. **11**(13): p. 1699-1701.

40. Francesko, A., et al., *GAGs-thiolated chitosan assemblies for chronic wounds treatment: control of enzyme activity and cell attachment.* Journal of Materials Chemistry, 2012. **22**(37): p. 19438-19446.

Chapter 6

Enzymatic functionalization of chitosan/gelatin hydrogels to modulate the proteolytic unbalance and bacterial growth in chronic wounds

Publications derived from this work: Guillem Rocasalbas, Antonio Francesko, Sonia Touriño, Xavier Fernández-Francos, Georg M. Guebitz and Tzanko Tzanov."Laccase-assisted formation of bioactive chitosan/gelatin hydrogel for chronic wound dressings" CARBPOL-D-12-01897-R1.

6.1. Introduction

The different chronic wound types do not share origin or cause, however they feature bacterial infection and high concentrations of matrix metalloproteases (MMPs), myeloperoxidase (MPO) and reactive oxidative species causing excessive degradation of the extracellular matrix (ECM) and the growth factors [1]. In healing wounds the MMPs are counteracted by their natural inhibitors [2], while in chronic wounds the ratio proteases/inhibitors is disturbed and most of these enzymes are uninhibited. The protease-antiprotease unbalance is further promoted by MPO-generated hypochlorous acid (HOCl), which from one sides inactivates the protease inhibitors and from another triggers the activity of latent MMP [3]. In addition, most chronic wounds are colonized with several bacterial species, e.g. S*taphylococcus aureus* and *Pseudomonas aeruginosa* [4].

Therefore, to stimulate the wound repair a dressing material should simultaneously: i) control the proteolytic and oxidative enzymes in the wound site, ii) provide a microorganism-free environment, and iii) maintain the tissue moisture while absorbing the excessive exudates. Biopolymers with intrinsic antimicrobial and/or healing promoting properties, such as chitosan and collagen/gelatin have been suggested for wound treatment, and though many of them are available on the market [5] only few are commercialized as chronic wound dressings. Chitosans of animal and fungal origin are linear and partly acetylated (1→4)-2-deoxy-β-D-glucans with intrinsic antimicrobial properties [6, 7]. On the other hand, gelatin (denatured collagen) besides ensuring the cell adhesion and growth [8], could serve as a competing substrate for several proteases present in the wound site thereby diverting them from digesting the ECM.

Chitosan and collagen/gelatin dressing materials are normally produced in the form of hydrogels according to the widely accepted concept of moist wound healing. The dressing stability in chronic wound application might be compromised by the requirement for low frequency of changing. Thus, these hydrogels need to feature biostability and mechanical strength achievable by additional crosslinking [9]. To this end, various chemical cross-linkers have been used, however, their cytotoxicity makes them unsuitable for biomedical applications [10]. The search for safe, natural crosslinking agents has brought about the use of genipin due to its low cytotoxicity [11] as well as plant polyphenolics, such as proanthocyanidins (PA) [12] and hydrolysable tannins (HyT) [13] among others for stabilization of carbohydrate/protein systems [14]. In the case of polyphenols, stabilization of carbohydrate and protein matrices is thought to be due to the physical interactions between these compounds and biopolymers. Such natural compound-based approaches can further be upgraded by the application of highly

specific enzymatic tools to achieve stable, covalently cross-linked gels. Oxidative enzymes, such as tyrosinase, peroxidase and laccase, are able to promote inter- and intra-molecular coupling reactions in biopolymers and natural phenolics [15-17]. For example, laccase (EC 1.10.3.2) would oxidize the phenolic compounds (PC) and tyrosine residues in proteins into reactive quinones, which can further react with nucleophiles such as amino groups from chitosan and gelatin by 1,4-Michael addition or Schiff base formation. On the other hand, PA and HyT are known for their antioxidant capacity, antimicrobial effect, anti-inflammatory and wound healing promoting properties [18]. Polyphenolic extracts from *Hamamelis virginiana* (Witch-hazel) rich in PA and HyT, are widely used in the therapy of skin diseases [19-21]. These extracts are able to protect cells from free radicals inhibiting the proliferation of melanoma cells [20] and exert an inhibitory effect over deleterious chronic wounds enzymes *in vitro* [22]. The therapeutic superiority of the extracts compared to the isolated single constituents at equivalent doses [23] was the reason to use as cross-linkers the natural extract instead of the single phenolic substances.

This study aims to generate hydrogel dressings for chronic wound application containing chitosan, gelatin, and natural phenolics further cross-linked by laccase to obtain bioactive and biostable materials with tunable physicomechanical and functional properties. Polyphenolic extract from *Hamamelis virginiana* will be oxidized by laccase in a one-step process under mild reaction conditions to covalently crosslink chitosan and gelatin. It is intended that the polyphenols play a dual role in the hydrogel: i) “passive” – being a structural element, and ii) “active” – modifying the chronic wound environment by attenuating the deleterious MMPs, MPO and ROS activities, and the bacterial infection.

6.2. Materials and methods

6.2.1. Reagents

Gelatin (G) was purchased from Fluka (France) and 90 kDa chitosan (C) from *Agaricus bisporus* was kindly supplied by KitoZyme (Belgium). Both C and G had pharmaceutical grade. *Hamamelis virginiana* (witch hazel) stems were provided by Martin Bauer GmbH (Germany).

Trametes sp. laccase (EC 1.10.3.2, Laccase L603P, 300 U/g of solid (activity measured at 45 ºC, pH 4.5, 1U: µmol of oxidized ABTS/min)) was purchased from Biocatalysts (UK). Collagenase from *Clostridium histolyticum* (ChC) (1676 U/mg solid, 1 U hydrolyses 1.0 µmol of FALGPA per minute at pH 7.5 and 25 ºC in the presence of Ca^{2+}) and N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala (FALGPA) collagenase substrate from Sigma-Aldrich (Spain) were used. Myeloperoxidase (MPO) from human leukocytes (1550 U/mg solid: 1 U will produce an increase in absorbance at 470 nm of 1.0 per minute at pH 7.0 and 25 °C, calculated from the initial rate of reaction using guaiacol as a substrate) comes from Planta Natural Products (Austria). Lysozyme from chicken egg white (dialyzed and lyophilized powder, 100000 units/mg solid (1 U corresponds to the amount of enzyme which decreases the absorbance at 450 nm by 0.001 per minute at pH 7.0 and 25 °C (*Micrococcus luteus*, ATCC 4698, as a substrate)) comes from Sigma-Aldrich (Spain). All other reagents used were of the highest grade commercially available from Sigma-Aldrich.

Cetrimide agar, Baird Parker agar and trypticase soy broth (TSB) were purchased from Sigma-Aldrich. Bacterial strains *Pseudomonas aeruginosa*, CECT 110T and *Staphylococcus aureus*, CECT 86T were provided by the Spanish Type Culture Collection (CECT) and grown in cetrimide and Baird Parker agar respectively.

6.2.2. Extraction and isolation of PC from Hamamelis virginiana

The phenolic extract from *H. virginiana* was obtained as previously reported in chapter 3.

6.2.3. Hydrogels preparation

Gelatin solution 2 % (w/v) was prepared in 25 mM succinate buffer at pH 4.5 and 60 °C. Chitosan was dissolved in 1 % HCl to obtain 2 % (w/v) solution, and the pH was adjusted to 4.5 with 1M NaOH. The solutions of chitosan and gelatin were mixed (C/G) at 2:3 ratio (w/w) at room temperature overnight. Thereafter, 11.25 mL of 1 % PC solution (w/v) in 25 mM succinate buffer pH 4.5 was added to 75 mL of the previously prepared C/G mixture 2:3 (w/w).

The crosslinking reaction was initiated by the addition of 1.5 mL of laccase solution (2 U/mL) and left to proceed for determined time periods up to 24 h at 45 °C under continuous stirring. The enzymatic reaction was terminated by heating at 100 ºC for 2 min and the resulting mixtures were cooled down to -80 °C and freeze-dried. The samples were designated according to the time of the enzymatic reaction. Control mixtures C/G-PC-C and C/G were prepared following the same procedure, but omitting the enzyme, or both PC and the enzyme (Table 6.1).

Table 6.1. Coding and reaction parameters of hydrogels.

Code Name	Initial concentration of PC in reaction mixture (µg GAE/ g hydrogel)a	Laccase incubation time (hours)
C/G	No PC	No laccase
C/G-PC-C	25500	No laccase
C/G-Lacc	No PC	4
C/G-PC-2h	25500	2
C/G-PC-4h	25500	4
C/G-PC-6h	25500	6
C/G-PC-8h	25500	8

[a]Gallic Acid Equivalents (GAE)

6.2.4. Hydrogels characterization

6.2.4.1. Rheological studies

Rheological measurements were carried out with an ARG2 rheometer (TA Instruments, UK) equipped with electrical heated plates. The samples were analyzed in parallel plate geometry (25 mm diameter) at 45 °C. The liquid samples were transferred to the preheated plate immediately after mixing the corresponding solutions for each test (time t = 0) and the measurements started at t = 60 s after thermal equilibration. The rheometer was operated in the oscillatory mode. A multiwave analysis program (1 Hz frequency, 3 and 5 Hz harmonics) with controlled 2 % strain was used to monitor the crosslinking process. The gelation time was determined using the G' and G" crossover criterion at 1 Hz, and as the frequency independent crossover of the tan δ curves at the different frequencies. To measure the mechanical properties of the resulting hydrogels, each experiment was repeated for defined reaction times, and the hydrogel was subjected to a stress sweep from 0.01 to 1000 Pa in the oscillatory mode at 1 Hz.

6.2.4.2. FTIR characterization of the chitosan/gelatin blends

Infrared spectra of the blends were collected over the 3500 - 800 cm^{-1} range using Perkin-Elmer Spectrum 100 (Perkin-Elmer, Massachusetts, USA) equipped with universal ATR sampling accessory, performing 50 scans for each spectrum.

6.2.4.3. Surface morphology of the hydrogels

Scanning electron microscopy (SEM) micrographs of the hydrogels with magnification x25 were obtained using a FESEM, Quanta 200 FEG (FEI Company, USA).

6.2.4.4. PC release from the hydrogels

The release of phenolics from the hydrogels was estimated according to the Folin-Ciocalteu method for determination of total phenol content in solution [24] by monitoring the reaction at 760 nm in a microplate reader (Infinite M200, Tecan, Austria) and the results were expressed in gallic acid equivalents (GAE). Freeze dried C/G-PC hydrogels were incubated in PBS at pH 7.4 and 37 °C for 60 h. At defined time intervals aliquots of the supernatant were removed, the total phenol content determined, and the phenolics release calculated according to equation 6.1.

Cumulative release (%) = PC_{rel}/PC_i x 100 **Equation 6.1.**

where PC_i is the total amount of PC and PC_{rel} is the amount of PC released in PBS.

6.2.4.5. Stability of the hydrogels

After reaching swelling equilibrium in PBS at 37 °C the hydrogels were removed from the medium, weighed and reintroduced to the medium. Thereafter, 1 mg/ml lysozyme was added. At regular time intervals, the hydrogels were removed from the medium and weighed. The medium was refreshed once a day. The results were compared with those obtained following the same procedure but omitting lysozyme. All experiments were performed in triplicate and the average weight loss value was calculated using equation 6.2.

Weight loss (%) = $(W_s - W_{Lys}) / Ws$ x 100 **Equation 6.2**

where W_S is the weight of the wet hydrogel at swelling equilibrium in PBS and W_{Lys} is the weight of the sample after lysozyme degradation.

6.2.5. Assessing in vitro the bioactivity of C/G-PC-2h

6.2.5.1. Hydrogel cleaning pre-treatment

In order to discriminate between the bioactivity due to released PC and the bioactivity of the hydrogel matrix itself, hydrogel samples were submitted to an extensive cleaning process to remove the non-covalently bonded PC. The hydrogels were incubated in PBS, changed several times, until no PC release was observed spectrophotometrycally. Afterwards the hydrogels were washed with Milli-Q H_2O to remove the salts from PBS, and then the samples were lyophilized. The cleaned hydrogel is refers in the text as hydrogel platform.

6.2.5.2. Radical scavenging activity

The radical scavenging activity of released PC and hydrogel platform was determined separately measuring the decrease in absorbance of 1,1-diphenyl-2-picrylhydrazyl radical (DDPH) at 515 nm [25]. All experiment were carried out in triplicate and the results of the assay were expressed relative to Trolox in terms of TEAC (Trolox equivalent antioxidant capacity) (mM Trolox eq/g hydrogel).

6.2.5.3. MPO inhibition

The inhibitory effect of the blends over MPO activity was measured using guaiacol as a substrate [26]. Hydrogel samples were incubated with 20 µL of MPO 0.325 µM (0.60 U) and 30 µL of guaiacol (167 mM) buffered with 450 µL PBS pH 7.4 at 37 °C. After predetermined incubation times 200 µL of the solution were placed in a 96-well microplate and the reaction was started by adding 22 µL of 1 mM H_2O_2. The change in absorbance at 470 nm was monitored during 5 cycles of 15 sec (75 sec.) using microplate reader Infinite M200 (Tecan, Austria). All measurements were carried out in triplicate and the activity was determined by the rate of absorbance increase per min and expressed as a percentage of enzyme inhibition compared to the control (MPO reaction mixture without sample).

6.2.5.4. Collagenase inhibition

The collagenase activity was measured using FALGPA substrate [27]. The collagenase was incubated in presence of hydrogels for different time of incubation. The hydrolysis of FALGPA with collagenase 0.2 µg/mL in 50 mM Tricine, containing 100 mM $CaCl_2$ and 400 mM NaCl, pH 7.4 was monitored spectrophotometrically at 345 nm during 5 min, immediately after mixing the substrate with the enzyme. All measurements were carried out in triplicate. The results were expressed as a percentage of enzyme inhibition compared to the control (sample without hydrogel).

To determine the kinetics of collagenase inhibition, the collagenase was treated with 0.125 mg/ml solution of PC for 3 h at 37 °C. FALGPA solutions (0.125 mM) were used as substrates in the different assays. The hydrolysis of FALGPA was monitored at 345 nm, immediately after the addition of PC incubated collagenase as done in the case of collagenase hydrogel inhibition. The kinetic parameters were calculated from Eisenthal-Cornish-Bowden plot and secondary plots of Kmapp/Vmaxapp vs [I] and 1/Vapp vs [I].

6.2.5.5. Protein adsorption on the hydrogels

Sorption studies were performed n 50 mM Tricine, 100 mM $CaCl_2$, 400 mM NaCl, pH 7.4. Hydrogel platforms were incubated with 40 μg/ml collagenase. The protein content in the supernatants was determined at defined time intervals over 24 h by using QuantiProTM BCA Assay Kit (Sigma).

6.2.5.6. Antimicrobial tests

Pseudomonas aeruginosa and *Staphylococcus aureus* were grown on Cetrimide Agar and Baird Parker Agar respectively at 37 °C for 24 h. A microorganism suspension in TSB of each strain was prepared at concentration of 10^4 CFU/mL. Then, the hydrogel samples (25 mg) were incubated under constant shaking with 25 ml of bacterial suspension in 50 mL cap sterile bottles at 37 °C for 24 h. Inoculated medium without sample was used as positive control, whereas as negative control uninoculated medium was used. To determine the inhibition of bacterial growth, at predetermined time intervals, the microorganism suspension (10 μL) was harvested in the agar plate, incubated 24 h at 37 °C and the grown CFU counted afterwards. The measurements were repeated three times for each sample and the average value of inhibition was calculated following equation 6.3

Inhibition (%) = $(CFU_S - CFU_C) / CFU_C \times 100$ **Equation 6.3.**

where CFU_s are the colonies grown in the bacterial suspension after 24 h incubation with hydrogel samples and CFU_C are the colonies grown in the bacterial suspension after 24 h incubation without sample. All measurements were carried out in triplicate and the mean values for bacteria inhibition are reported.

6.3. Results and discussion

6.3.1. Enzymatic cross-linking and functionalization of chitosan/gelatin hydrogels

The laccase-catalyzed gel formation in chitosan, gelatin and PC formulation was evaluated in situ by means of rheological measurements. To investigate the influence of PC and laccase alone on the gelation, mixtures of chitosan/gelatin/laccase (C/G-Lacc) and chitosan/gelatin/PC (C/G-PC-C) were monitored as controls. There was a slow evolution of the storage modulus (G

the completion of the cross-linking process. Laccase induced oxidation of tyrosine residues into tyrosyl radicals that form primarily isodityrosine and dityrosine bonds or their oxidation to quinones [28] allows them to undergo various coupling reactions with nucleophilic groups present in chitosan and gelatin [29]. Nevertheless, the laccase effect was rather limited in the cross-linking of chitosan and gelatin due to the low tyrosine content in gelatin (about 0.5 %) [30]. Thus, the cross-linked network is not enough developed to create a stable hydrogel.

Finally, the storage modulus (G'), monitored in the rheological evolution of the C/G with PC and laccase, increased rapidly upon addition of laccase to C/G and PC mixtures, indicating crosslinking between O-quinones formed by the enzymatic oxidation of PC and the nucleophilic groups present in the biopolymers. The gelation point indicated the formation of an incipient weak elastic network with a low cross-linking density. Gelation took place after 94 min for the C/G-PC system (Figure 6.1.A). G' increased steadily afterwards due to further increase of the cross-linking density. The loss modulus (G") also increased due to the increase in the molecular weight caused by the crosslinking reaction. The relative magnitude between storage and loss moduli is given by tan δ (tan δ =G´´/G´), being tan δ the phase angle. Since tan δ < 1, the elastic solid behavior of the samples was predominant over their viscous liquid behavior. The decrease of tan δ confirmed the progressive cross-linking of the hydrogel. G' leveled off after 500 min showing completion of the cross-linking process Beyond this point, G' decreased and tan δ increased corresponding to a failure of the cross-linked structure due to brittleness and excessive strain. The water evaporation observed might have contributed to the failure of the gel network and subsequent loss of hydrogel properties.

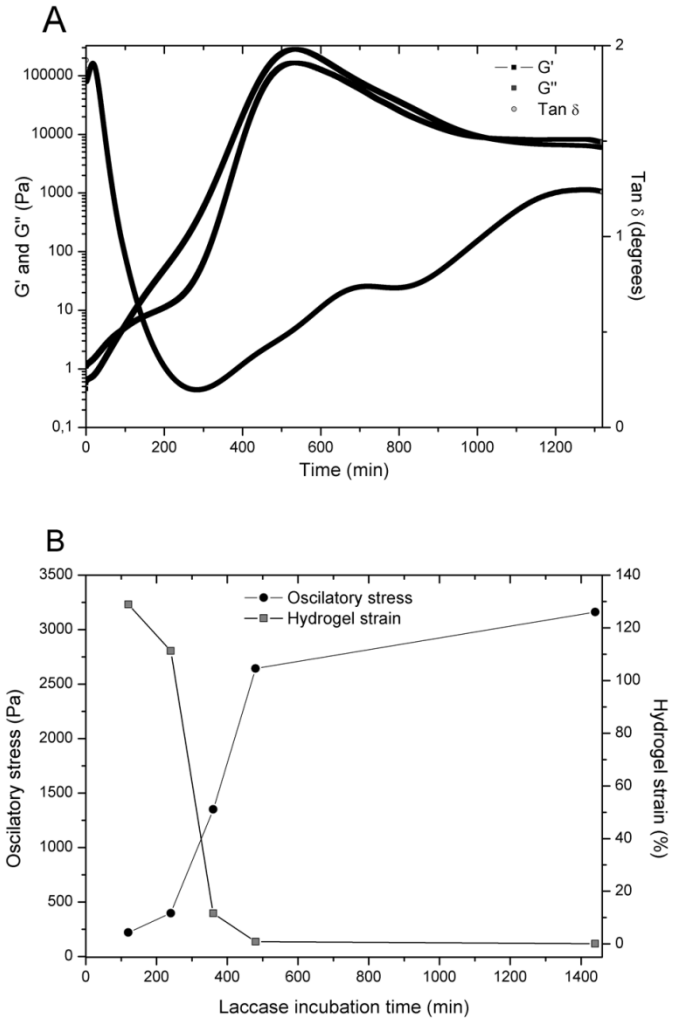


Figure 6.1. Rheological characterization of the laccase-assisted cross-linking reaction. C/G (2:3, % wt), laccase (2 U/ml) and PC (1.5 mg/ml) in succinate buffer (25 mM, pH 4.5) at 45 °C. (A) Storage modulus (G'), loss modulus (G'') and tan δ evolution, (B) Oscillation stress sweep–strain curves of C/G-PC hydrogels after different reaction times (2, 4, 6, 8 and 24 h).

Unlike the C/G-Lacc control, the mixture of C/G, PC and laccase showed higher gelation point, probably owing to polymerization reactions of PC [31] retarding the cross-linking . On the other hand the completion of the cross-linking process after 500 min of enzymatic reaction with G' values higher than 10^5 Pa pointed out a high cross-linking density, thereby confirming that the laccase activation of PC was a major factor driving the biopolymer blends to gelation.

The maximum stress and strain endured by the hydrogel before the network failure were determined on the basis of stress sweeps after different reaction times. The stress and strain increased and decreased respectively with the increase of the laccase reaction time as a consequence of the increased cross-link density of the chitosan, gelatin and PC network (Figure

6.1.B). The changes of stress and strain were more pronounced during the first 8 h, after which the rate of increase of the oscillatory stress was considerably slower. These results could be explained by reaching the maximum cross-link density after 500 min of enzymatic reaction. Longer treatment time (up to 24 h) did not bring about considerable increase of hydrogel stress. Moreover, the samples prepared in a reaction longer than 8 h were stiff and brittle, and were not considered for further investigations.

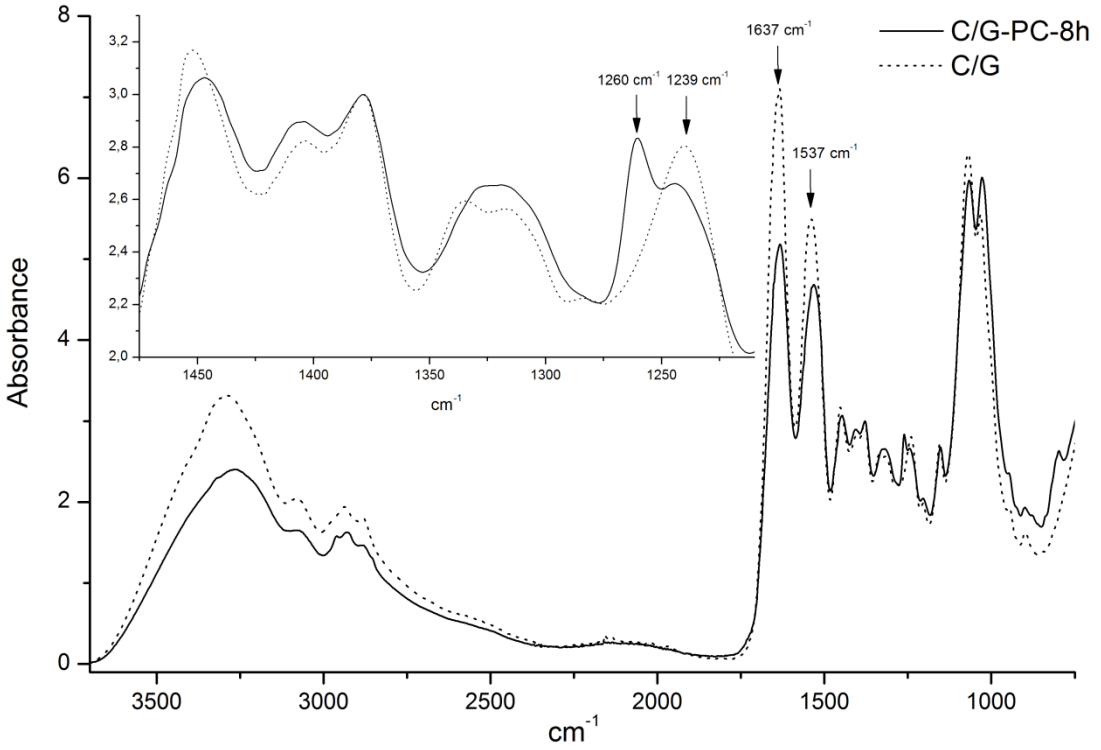


Figure 6.2. Normalized FTIR spectra of freeze-dried C/G and C/G-PC-8h samples. The inset image magnifies the region from 1500 cm-1 to 1200 cm^{-1}

The complexity of the used enzymatic cross-linking approach may involve a broad range of molecular interactions and reactions, including electrostatic attractions, hydrogen-bonding, PC polymerization and, above all, the covalent linkages between C/G systems and laccase-activated PC. Clear differences were found in the spectrum of the control sample C/G comparing to sample C/G-PC-8h with the highest cross-link density (Figure 6.2). Characteristic bands at 1637 cm^{-1} (amide I) and 1537 cm^{-1} (–NH_2 bending) were observed for both samples. For C/G-PC-8h the peak intensity of unreacted amino groups at 1537 cm^{-1} as well as amide I peak at 1637 cm^{-1} decreased compared to the control, indicating the involvement of these groups in the cross-

linking reaction. Additionally, a new peak at 1260 cm^{-1} appeared in the region of 1215 – 1270 cm^{-1} attributed to C-N stretching of aryl amides formed via Michael addition, whereas the peak at 1239 cm^{-1} attributed to alkyl amines decreased. The new bands at 789 cm^{-1}, 837 cm^{-1} and 875 cm^{-1} could be assigned to different pattern substitution of the aromatic rings [32]. Therefore, the FTIR spectra confirmed that the phenolic compounds from the *H. virginiana* extract were covalently incorporated into the C/G network and the cross-linking occurred most probably and predominantly via Michael addition.

The cross-sectional SEM micrographs (Figure 6.3) of representative freeze-dried C/G-PC hydrogels revealed porous structures with different pore size and shape depending on the cross-linking density (time of enzymatic reaction). Hydrogen bonding and ionic hydrophobic interactions between PA and biopolymers led to a sheet-like structure of the non-cross-linked control sample [33, 34]. On the contrary, the enzymatically cross-linked hydrogels presented a porous structure with increasing pore size as a function of the extent of cross-linking, also observed in similar cross-linked biopolymer systems [9].

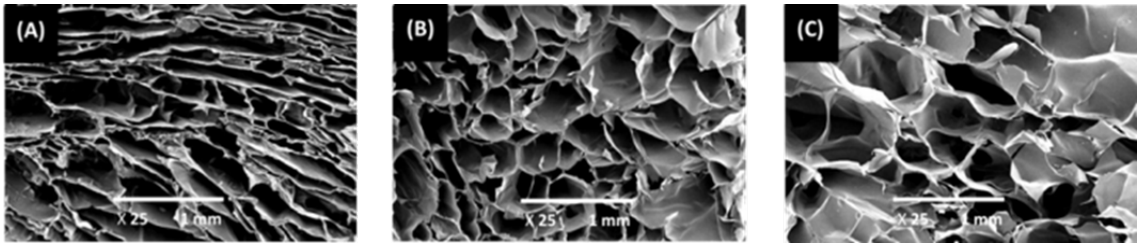


Figure 6.3. Scanning electron micrographs of freeze-dryed C/G-PC hydrogels. Magnification x25. A) C/G-PC-C; B) C/G-PC-4h and C) C/G-PC-8h

6.3.2. Stability of the hydrogels

The stability of the hydrogel at use in chronic wound management is an important parameter due to the requirement for less frequent changing of the dressings. The non-cross-linked control sample C/G-PC-C was completely disintegrated after 1 h in PBS, unlike the recently reported 5-days stability and pepsin digestibility of ionically formed chitosan/gelatin/PC films in water [34]. Similar to C/G-PC-C, the other control sample C/G-Lacc was also completely solubilized after 1h in PBS, confirming the poor cross-linking density in such system. On the contrary, all enzymatically cross-linked PC-containing hydrogels retained more than 80 % of their weight after 60 h in PBS (Figure 6.4). Thus, the laccase-assisted cross-linking of the hydrogels with PC

was a prerequisite for their stability in physiological conditions. Since the main enzyme in wound fluids that could hydrolyze the β(1→4) glycosidic linkage in chitosan is the lysozyme [35], the hydrogel stability against lysozyme digestion was further evaluated. As expected, comparing the stability of the hydrogels in the presence and absence of the enzyme, faster degradation due to the lysozyme hydrolysis of the chitosan component was observed for all experimental groups (Figure 6.4A-C).

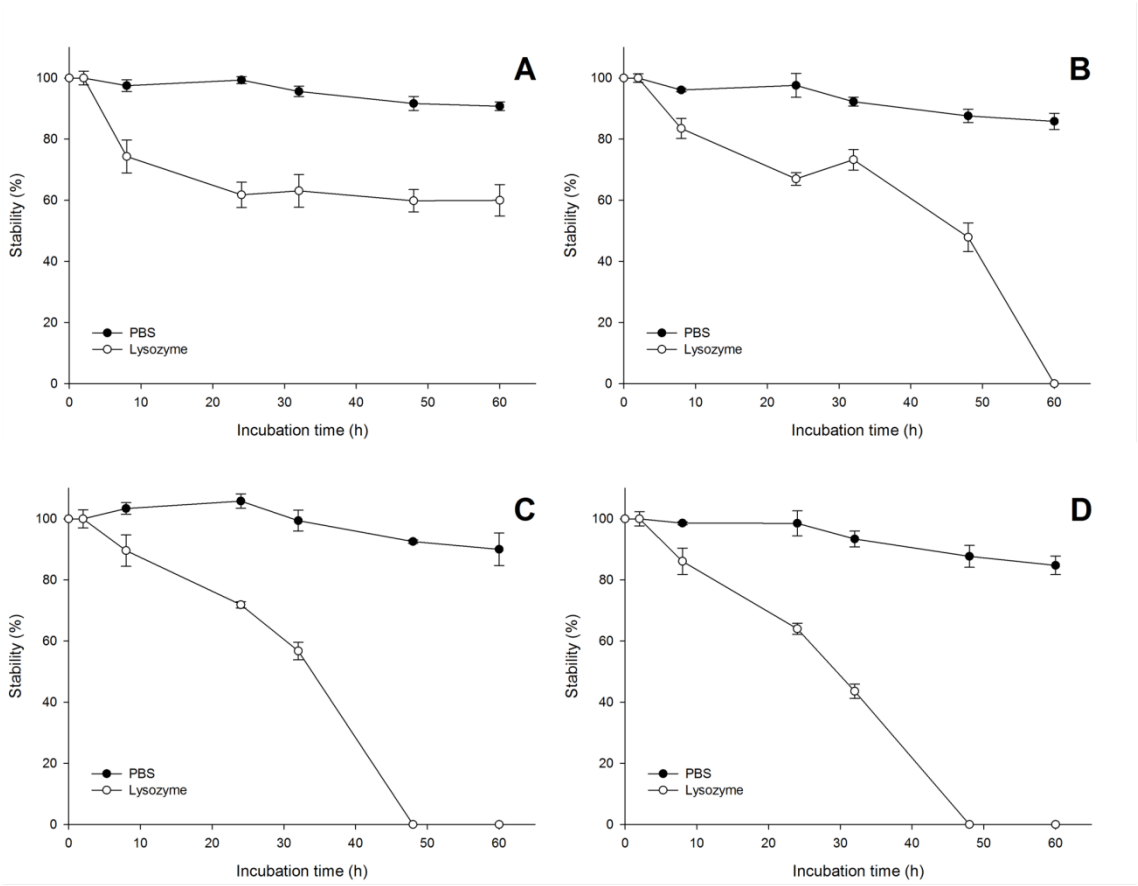


Figure 6.4. Stability of hydrogels in PBS and PBS with lysozyme (1 mg/mL) at 37 °C as a function of time. (A) C/G-PC-2h ; (B) C/G-PC-4h; (C) C/G-PC-6h and (E) C/G-PC-8h. At regular time intervals, the hydrogels were removed from the medium and weighed. The medium was refreshed once a day. The results are expressed as the average of the hydrogel weight loss (stability) and are mean values from three replicates.

Surprisingly, the degree of hydrogel degradation by lysozyme was inversely proportional to the laccase incubation time. Usually, the cross-linking should stabilize the hydrogel [36]. Samples C/G-PC-6h and C/G/PC-8h were completely degraded by lysozyme after 48 h incubation

(Figure 6.4D), whereas C/G-PC-2h preserved its structural integrity even after 60 h (Figure 6.4A). Considering that the composition of *H. virginiana* extract used in the present study is rich in monomeric and oligomeric phenolic structures [20], these unexpected results could be explained by the capacity of low molecular weight PC to inactivate lysozyme as recent studies revealed [37]. Shorter laccase application ensures a higher amount of non-covalently loaded low molecular weight PC, while longer incubation time results in higher cross-linking between PC and C/G and further polymerization of PC [38]. Consequently, the lower availability of non-grafted, oligomeric PC in the increasingly cross-linked hydrogels would, lead to higher lysozyme degradability.

6.3.3. PA release

Our previous studies demonstrated the antioxidant activity and inhibitory effect on MPO and collagenase of *H. virginiana* PC, required for the initiation of the wound healing process [22]. Thus, besides stabilization of the hydrogel matrix, the PC from *H. virginiana* were expected to act as active agents, which controlled release would stimulate the healing process [39]. The cumulative release of PC from the hydrogels obtained in this study showed two phases: an initial burst release during the initial hydrogel swelling, followed by a sustained release (Figure 6.5). Shorter laccase cross-linking treatment results in higher amount of non-covalently grafted PC and subsequently higher PC release. The sample C/G-PC-2h, showing the highest PC release and stability, was selected to study the bioactivity of the hydrogels. Replicates of C/G-PC-2h were submitted to an extensive cleaning process to remove the non-covalently bonded PC in order to distinguish the effect of the permanently modified with PC hydrogel platform. The following experiments were conducted with both C/G-PC-2h and cleaned C/G-PC-2h hydrogel platform (sample further designated as “C/G-PC-2h hydrogel platform” in the text).

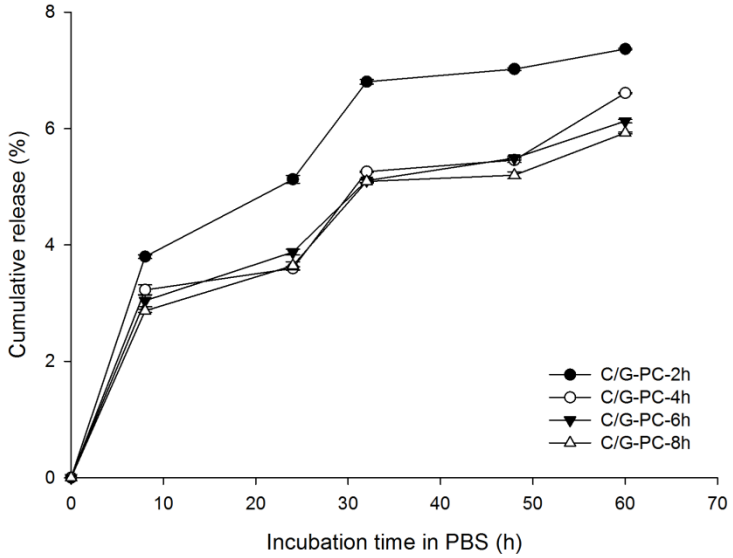


Figure 6.5. *In vitro* cumulative release of non-covalently loaded PA from the hydrogels. The samples were incubated in PBS, pH 7.4 at 37 °C for 60 h. At defined time intervals aliquots of the supernatant were removed and the total phenol content determined. The results are averaged from three replicates.

6.3.4. In vitro assessment of the bioactivity of the hydrogels

6.3.4.1. Radical scavenging activity

C/G-PC-2h hydrogel platform showed considerably higher radical scavenging activity compared to the activity of the released PC (Table 6.2), accounting for the low liberation of PC (only 4 % in 24 h) from the hydrogel (Figure 6.5). As expected, the antioxidant activity of the released PC measured at predefined time intervals (1 – 24 h) increased gradually with time.

At the same time the activity of the C/G-PC-2h hydrogel platform increased as well possibly due to swelling of the hydrogel allowing higher accessibility of DPPH to react with the covalently incorporated in the bulk of the hydrogel PC. PA efficiently scavenged ROS both upon release and acting from the biopolymer matrix.

Table 6.2. DPPH radical scavenging activity of released PC and C/G-PC-2h hydrogel platform. The radical scavenging activity was determined measuring the decrease in absorbance of DPPH radical at 515 nm. The results represent mean values from three replicates ± standard deviation

	TEAC (mM Trolox/g hydrogel)	
Incubation time	**Released PC**	**C/G-PC-2h hydrogel platform**
1 h	1.78 ± 0.01	54.70 ± 9.11
2 h	2.00 ± 0.01	57.00 ± 4.42
6 h	2.02 ± 0.00	58.32 ± 4.18
8 h	2.05 ± 0.01	56.35 ± 9.12
24 h	2.10 ± 0.01	59.09 ± 2.20

6.3.4.2. Inhibition of chronic wound enzymes

Chronic inflammation leads to the accumulation of MPO-generated HOCl able to oxidize most biological molecules. In addition HOCl triggers the MMPs activation. [10]. The stimulated proteolytic activity contributes to ECM degradation and chronicity of the wounds. It is therefore crucial to control the MPO and MMPs activities in order to provide conditions for healing and ECM reconstruction. The levels of HOCl can by modulated by: i) MPO substrates diverting the enzyme from generating HOCl, and ii) HOCl scavengers.

In this study, both C/G-PC-2h and C/G-PC-2h hydrogel platform inhibited the collagenase activity (higher inhibition with C/G-PC-2h) during the first 8 h of incubation, followed by a sustained inhibitory effect up to 24 h. Moreover, the profile of the collagenase inhibition curve in presence of C/G-PC-2h hydrogel platform was similar to the collagenase adsorption curve (Figure 6.6.A and Figure 6.6.B) suggesting that enzyme protein adsorption on the hydrogels partially inactivates the collagenase. On the other hand, the higher inhibitory efficiency of C/G-PC-2h sample could be related to the released PC [22]. A dose-dependent collagenase inhibition was reported for different plant polyphenol extracts. Interaction of the phenolic molecules with the enzymes was suggested to induce conformational changes or blocking of the enzyme active site [40].

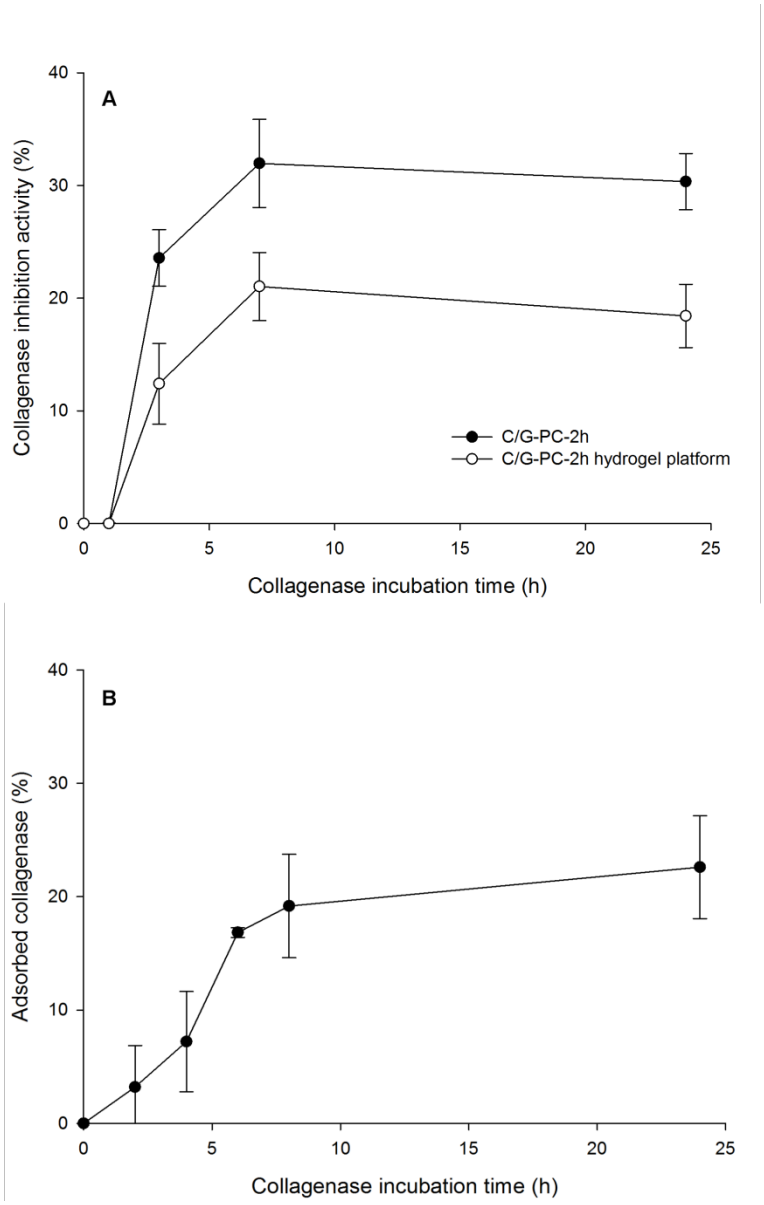


Figure 6.6. Attenuation of collagenase activity in the presence of PC-cross-linked hydrogels. **A**) Inhibition of collagenase activity in the presence of PC-cross-linked hydrogels. The samples and the enzyme (0.2 µg/mL) were incubated in 0.05 M Tricine buffer pH 7.5 (with 0.4 M NaCl and 0.01 M $CaCl_2$, at 37 °C for 3, 6 and 24 h. The reaction was initiated by the addition of FALGPA (300 mM) and the decrease of absorbance at 324 nm was monitored during 5 min to detect FALGPA hydrolysis products. **B**)Collagenase adsorption on C/G-PC-2h hydrogel platform. The samples and the enzyme were incubated in 0.05 M Tricine buffer pH 7.5 (with 0.4 M NaCl and 0.01M $CaCl_2$, at 37 °C for 24 h. The protein content in the supernatant was measured at predetermined incubation time using QuantiProTM BCA Assay Kit. All results represent mean values of three replicates ± standard deviation

To analyze the inhibition of collagenase by released PC, its activity was studied in presence of different concentrations of PC and FALGPA. The direct-linear Eisenthal-Cornish-Bowden plot

(Figure 6.7) describing the kinetics of collagenase inhibition by PC revealed a mixed-type inhibition mechanism.

The kinetic collagense-PC dissociation constant (Kic) and collagenase-FALGPA-PC dissociation constant (Kiu) (Cornish-Bowden notation) were calculated by secondary plots of Kmapp/Vmaxapp vs [PC] and 1/Vapp vs [PC] and the Kic and Kiu values obtained were 6.1580 mM and 0.3904 mM respectively. These data suggest that the released PC bind both to collagense and the collagenase–FALGPA complex probably promoting conformational changes in the enzyme structure and inhibiting its activity [41]. Mixed-type inhibition may be considered as a consequence of the presence of different phenolic compounds in *Hamamelis virginiana* extract acting by different inhibition mechanisms [40-42]. These experiments indicate that the proteolytic activity regulation by the developed hydrogels was due both to release of PC and adsorption of the enzyme protein on the biopolymer matrix.

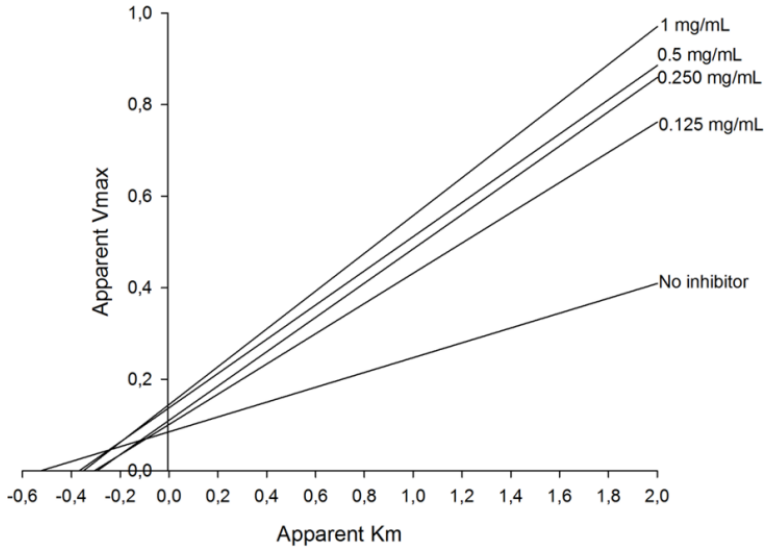


Figure 6.7. Direct-linear Eisenthal-Cornish-Bowden plots of the inhibition of collagenase at various fixed concentrations of PC with FALGPA as a substrate. The enzyme (0.2 µg/mL) was incubated in 0.05 M Tricine buffer pH 7.5 (with 0.4 M NaCl and 0.01M CaCl2, at 37 °C for 3 h) in presence of different collagenase concentrations. The reaction was initiated by addition of four different concentrations of FALGPA for each concentration of PA and the decrease of absorbance at 324 nm was monitored during 5 min to detect FALGPA hydrolysis products. The results represent mean values of three replicates.

In a next step the MPO inhibitory capacity of C/G-PA-2h and C/G-PC-2h hydrogel platform was measured. The C/G-PC-2h hydrogel platform did not show any MPO inhibition, while the C/G-PC-2h caused about 95 % enzyme inhibition within 10 min, further maintained up to 3 h and then followed by a decrease to 79.7 % in 24 h (Table 6.3).

Table 6.3. Myeloperoxidase (MPO) inhibitory activity. C/G-PC-2h was incubated with 0.60 U MPO in the presence of guaiacol (final concentration 10 mM) buffered with PBS pH 7.4 at 37 °C. After predetermined incubation times MPO activity was measured and expressed as a percentage of enzyme inhibition compared to the control (MPO reaction mixture without sample). The results are mean values of three replicates ± standard deviation.

Sample	MPO incubation time (min)	MPO Inhibition activity (%)
C/G-PC-2h	10	95.66 ± 0.58
	30	95,93 ± 0.19
	60	95,55 ± 0.28
	180	94,84 ± 0.36
	360	88.00 ± 2.13
	1440	79.70 ± 0.61
C/G-PC-2h hydrogel platform	10-1440	No inhibition

The inhibition of MPO was due to the released PC acting as inhibitors and competing substrates for MPO in the enzyme activity assay using guaiacol [22]. If translated to the chronic wound environment, this combined MPO inhibition and the diversion of the enzyme from its natural cycle would lead to the attenuation of HOCl levels and consequently allow the healing to progress.

6.3.4.3. Antibacterial studies

The antimicrobial activity of the enzymatically-assembled hydrogels was evaluated against the Gram-positive *Staphylococcus aureus* and the Gram-negative *Pseudomonas aeruginosa*. The hydrogels inhibited the growth of both strains after 24 h incubation (Table 6.4). The inhibition of *S. aureus* growth was more pronounced than that of *P. aeruginosa* (respectively 85 and 45 % for the C/G-PC-2h, and 53 and 23 % for C/G-PC-2h hydrogel platform). The tolerance of bacteria for polyphenols depends on the bacteria species and molecular structure of the polyphenols. The extract of *Hamamelis virginiana* is rich in epicatechins (EC) which possess antimicrobial activity against *S. aureus* [43] and epigallocatechin gallates (EGCG) that exert antimicrobial activity against *P. aeruginosa* [44] through perturbation of the cell membrane. The higher antibacterial activity observed for C/G-PC-2h was probably due to the release of non-covalently adsorbed PC containing both EC and EGCG. On the other hand, the intrinsic antibacterial properties chitosan are believed to be determined by interactions between its cationic amino groups and the negatively charged microbial cell membranes, resulting in alteration of membrane wall permeability. This mechanism has been reported to be common for both strains, though more efficient on the cell membrane of *S. aureus* [45]. Therefore, the

antibacterial capacity of the chitosan/gelatin/PC hydrogels is a result of the combined action of the biopolymer platforms, covalently attached PC and released PC.

Table 6.4. Inhibitory effect of hydrogels after 24 h incubation with *S. aureus* and *P. aeruginosa.* The hydrogels were incubated in TSB bacterial suspension of each strain (104 CFU/ml) for 24 h at 37 ºC. The results are mean values of three replicates ± standard deviation.

	Inhibition of bacterial growth (%)	
	Staphylococcus aureus	***Pseudomonas aeruginosa***
C/G-PC-2h	84.32 ± 7.56	37.06 ± 6.34
C/G-PC-2h hydrogel platform	65.31 ± 2.12	23.59 ± 1.03

6.4. Conclusions

Laccase-assisted coupling between polyphenols of *Hamamelis virginiana* and primary amino groups in chitosan and gelatin was used as a versatile functionalization method to obtain stable under physiological conditions bioactive hydrogels. The laccase-initiated oxidation of phenolics resulted in a biopolymer network stabilized by quinones /amino coupling reactions predominantly via Michael addition mechanism between the phenolic compounds and the polymers. The phenolic compounds loaded on the hydrogels exerted both: i) structural function stabilizing the hydrogel, and ii) bio-activity inhibiting deleterious wound enzymes and bacterial growth to stimulate the wound healing process. The physico-mechanical properties of the chitosan/gelatin-PC hydrogels were dependent on the duration of the enzymatic reaction. Hydrogels stable under physiological conditions and resistant to lysozyme degradation were obtained with relatively short enzymatic reaction time (2h). The inhibition of the deleterious chronic wound enzymes largely depended on the amount of the released phenolic compounds, being responsible for the inhibition of myeloperoxidase activity acting as enzyme substrates. On the other hand, partial inhibition of collagenase activity was due to both released phenolic compounds and enzyme protein adsorption onto the hydrogel platform. In addition, the enzymatically-assembled hydrogels inhibited the bacterial growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* commonly found in chronic wound.

6.5. References

1. Trengove NJ, S.M., MacAuley S et al., *Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors.* Wound Repair and Regeneration, 1999. **7**: p. 442–452.
2. Taylor, K.B., et al., *The mechanism of inhibition of collagenase by TIMP-1.* The Journal of Biological Chemistry, 1996. **271**(39): p. 23938-45.
3. Sorsa, T., et al., *Collagenase in synovitis of rheumatoid arthritis.* Seminars in Arthritis and Rheumatism, 1992. **22**(1): p. 44-53.
4. Fazli, M., et al., *Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds.* Journal of Clinical Microbiology, 2009. **47**(12): p. 4084-9.
5. Muzzarelli, R.A.A., *Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone.* Carbohydrate Polymers, 2009. **76**(2): p. 167-182.
6. Muzzarelli, R.A.A., *Chitin*. 1977: Pergamon Press.
7. Muzzarelli, R.A.A., et al., *Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial.* Carbohydrate Polymers, 2012. **87**(2): p. 995-1012.
8. Pulieri, E., et al., *Chitosan/gelatin blends for biomedical applications.* Journal of Biomedical Materials Research Part A, 2008. **86A**(2): p. 311-322.
9. Moura, M.J., et al., *In Situ Forming Chitosan Hydrogels Prepared via Ionic/Covalent Co-Cross-Linking.* Biomacromolecules, 2011. **12**(9): p. 3275-3284.
10. Fu, X., et al., *Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase.* The Journal of Biological Chemistry, 2001. **276**(44): p. 41279-87.
11. Muzzarelli, R.A.A., *Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids.* Carbohydrate Polymers, 2009. **77**(1): p. 1-9.
12. Kim, S., et al., *Chitosan/gelatin-based films crosslinked by proanthocyanidin.* Journal of Biomedical Materials Research. Part B, Applied Biomaterials, 2005. **75**(2): p. 442-50.
13. Shimada, T., et al., *Role of tannin-binding salivary proteins and tannase-producing bacteria in the acclimation of the Japanese wood mouse to acorn tannins.* Journal of Chemical Ecology 2006. **32**(6): p. 1165-80.
14. Van Vlierberghe, S., P. Dubruel, and E. Schacht, *Biopolymer-Based Hydrogels As Scaffolds for Tissue Engineering Applications: A Review.* Biomacromolecules, 2011. **12**(5): p. 1387-1408.
15. Moreira Teixeira, L.S., et al., *Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering.* Biomaterials, 2012. **33**(5): p. 1281-1290.
16. Mikolasch, A. and F. Schauer, *Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials.* Applied Microbiology and Biotechnology, 2009. **82**(4): p. 605-624.

17. Muzzarelli, R.A.A., et al., *Tyrosinase-mediated quinone tanning of chitinous materials.* Carbohydr. Polym., 1994. **24**(4): p. 295-300.

18. Quideau, S., et al., *Plant Polyphenols: Chemical Properties, Biological Activities, and Synthesis.* Angewandte Chemie International Edition, 2011. **50**(3): p. 586-621.

19. Deters, A., et al., *High molecular compounds (polysaccharides and proanthocyanidins) from Hamamelis virginiana bark: influence on human skin keratinocyte proliferation and differentiation and influence on irritated skin.* Phytochemistry, 2001. **58**(6): p. 949-58.

20. Touriño, S., et al., *Highly galloylated tannin fractions from witch hazel (Hamamelis virginiana) bark: electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells.* Chem. Res. Toxicol., 2008. **21**(3): p. 696-704.

21. Lizárraga, D., et al., *Witch Hazel (Hamamelis virginiana) Fractions and the Importance of Gallate Moieties□Electron Transfer Capacities in Their Antitumoral Properties.* J. Agric. Food Chem., 2008. **56**(24): p. 11675-11682.

22. Díaz-González, M., et al., *Inhibition of deleterious chronic wound enzymes with plant polyphenols.* Biocat. Biotrans., 2012. **30**(1): p. 102-110.

23. Wagner, H. and G. Ulrich-Merzenich, *Synergy research: Approaching a new generation of phytopharmaceuticals.* Phytomedicine, 2009. **16**(2–3): p. 97-110.

24. Scalbert, A., B. Monties, and G. Janin, *Tannins in wood: comparison of different estimation methods.* Journal of Agricultural and Food Chemistry, 1989. **37**(5): p. 1324-1329.

25. Brand-Williams, W., M.E. Cuvelier, and C. Berset, *Use of a free radical method to evaluate antioxidant activity.* LWT - Food Science and Technology, 1995. **28**(1): p. 25-30.

26. Capeillere-Blandin, C., *Oxidation of guaiacol by myeloperoxidase: a two-electron-oxidized guaiacol transient species as a mediator of NADPH oxidation.* Biochemical Journal, 1998. **336 (Pt 2)**: p. 395-404.

27. Van Wart, H.E. and D.R. Steinbrink, *A continuous spectrophotometric assay for Clostridium histolyticum collagenase.* Analytical Biochemistry, 1981. **113**(2): p. 356-365.

28. Gerrard, J.A., *Protein–protein crosslinking in food: methods, consequences, applications.* Trends in Food Science & Technology, 2002. **13**(12): p. 391-399.

29. Mattinen, M.-L., et al., *Laccase-catalyzed polymerization of tyrosine-containing peptides.* FEBS Journal, 2005. **272**(14): p. 3640-3650.

30. Eastoe, J.E., *The amino acid composition of mammalian collagen and gelatin.* Biochemical Journal, 1955. **61**(4): p. 589-600.

31. Mita, N., et al., *Laccase-Catalyzed Oxidative Polymerization of Phenols.* Macromolecular Bioscience, 2003. **3**(5): p. 253-257.

32. Cui, Y.L., et al., *Biomimetic surface modification of poly(L-lactic acid) with chitosan and its effects on articular chondrocytes in vitro.* Biomaterials, 2003. **24**(21): p. 3859-68.

33. Wang, Y., et al., *A small-angle neutron scattering and rheology study of the composite of chitosan and gelatin.* Colloids and Surfaces B: Biointerfaces, 2009. **70**(2): p. 254-258.

34. Kim, S., et al., *Chitosan/gelatin-based films crosslinked by proanthocyanidin.* Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2005. **75**(2): p. 442-50.

35. Hjerde, R.J.N., et al., *Chemical composition of O-(carboxymethyl)-chitins in relation to lysozyme degradation rates.* Carbohydrate Polymers, 1997. **34**(3): p. 131-139.

36. Zhang, H., A. Qadeer, and W. Chen, *In Situ Gelable Interpenetrating Double Network Hydrogel Formulated from Binary Components: Thiolated Chitosan and Oxidized Dextran.* Biomacromolecules, 2011. **12**(5): p. 1428-1437.

37. Guzzo, F., et al., *The inhibitory effects of wine phenolics on lysozyme activity against lactic acid bacteria.* International Journal of Food Microbiology, 2011. **148**(3): p. 184-190.

38. Desentis-Mendoza, R.M., et al., *Enzymatic polymerization of phenolic compounds using laccase and tyrosinase from Ustilago maydis.* Biomacromolecules, 2006. **7**(6): p. 1845-54.

39. Hori, K., et al., *Controlled-release of epidermal growth factor from cationized gelatin hydrogel enhances corneal epithelial wound healing.* Journal of Controlled Release, 2007. **118**(2): p. 169-176.

40. Kim, Y.-J., H. Uyama, and S. Kobayashi, *Inhibition effects of (+)-catechin–aldehyde polycondensates on proteinases causing proteolytic degradation of extracellular matrix.* Biochemical and Biophysical Research Communications, 2004. **320**(1): p. 256-261.

41. Ganesan, K., et al., *Protective Effect of Withania somnifera and Cardiospermum halicacabum Extracts Against Collagenolytic Degradation of Collagen.* Applied Biochemistry and Biotechnology, 2011.

42. Madhan, B., et al., *Role of green tea polyphenols in the inhibition of collagenolytic activity by collagenase.* International Journal of Biological Macromolecules, 2007. **41**(1): p. 16-22.

43. Masika, P.J., N. Sultana, and A.J. Afolayan, *Antibacterial Activity of Two Flavonoids Isolated from Schotia latifolia.* Pharmaceutical Biology, 2004. **42**(2): p. 105-108.

44. Xie, W., P. Xu, and Q. Liu, *Antioxidant activity of water-soluble chitosan derivatives.* Bioorg. Med. Chem. Lett., 2001. **11**(13): p. 1699-1701.

45. Tao, Y., L.-H. Qian, and J. Xie, *Effect of chitosan on membrane permeability and cell morphology of Pseudomonas aeruginosa and Staphyloccocus aureus.* Carbohydr. Polym., 2011. **86**(2): p. 969-974.

Chapter 7

Conclusions and future remarks

7.1. Conclusions

Abundant levels of ROS and deleterious and oxidative enzyme as MMPs and MPO are the main responsible to impair the normal wound healing process leading to the formation of chronic wounds. In addition a major complication of wound healing is the occurrence of bacterial infections. Ideally, because of the multifactorial nature of chronic wounds, the therapeutic wound healing approach should comprise wound dressings that possess the ability to directly and indirectly modulate the biochemical environment of the chronic wounds beyond providing physical protection and optimal moisture environment to encourage the healing process.

To this end, different approaches were successfully developed during this thesis. A new method was developed for permanent functionalization of chitosan platform with active agents from *H. virginiana* via thiolysis reaction and a new laccase-mediated cross-linked approach to generate chitosan/gelatin hydrogel dressings.

The work developed in this thesis can be divided into two main parts: The first part consist in the identification and study of biopolymer platforms and potential active agents (chapters 2 and 3) and the second part consists in the development of potential wound dressings materials (chapter 4 and 5). The general conclusions of this research are summarized as follows:

Identification and evaluation of functionalized thiolated chitosan and *Hamamelis virginiana* extracts as a chronic wound healing promoters

The chitosan functionalized with thiol moieties was found to be efficient in the inhibition of collagenase, making it a suitable platform for wound dressings. In addition the combination of -NH_2 and -SH chemistry allows to graft further other active agents onto thiolated chitosan platform, increasing its multi-functionality.

Plant polyphenols from *H. virginiana* were found to have both a strong antioxidant activity and an inhibitory effect on MPO and collagenase – features potentially beneficial for the wound healing process. As chronic ulcer fluids contain elevated levels of ROS and neutrophil-derived deleterious oxidative and proteolytic enzymes, the use of thiolated chitosan as biopolymer platform as well as the application of extract from *H. virginiana* seems to be a promising approach for use in chronic wounds management.

7.1. Conclusions

Development of a new method for permanent for functionalization of chitosan platform with active agents from *Hamamelis virginiana* extracts

Thiolysis reaction has been used innovatively as a synthetic approach for permanent grafting of phenolic antioxidants on chitosan. The resulted biopolymer platform combines multiple functional groups, such as amino, thiol, imino and phenolic moieties. The new chitosan conjugate showed increased antioxidant activity in comparison with the non-functionalized chitosan and thiolated chitosan. Furthermore, the new conjugate showed increased antimicrobial efficiency against the Gram negative *Pseudomonas aeruginosa*.

The second approach focused on the use of laccase-assisted coupling between phenolic moieties of *Hamamelis virginiana* proanthocyanidins with chitosan and gelatin as a functionalization method to obtain stable ad bioactive hydrogels. *Hamamelis virginiana* extract was oxidized by laccase in a one-step process under mild reaction conditions to covalently crosslink chitosan and gelatin. Polyphenols played a dual role in the hydrogel: i) “passive” – being a structural element, obtain stable and bioactive hydrogels and ii) “active” – modifying the chronic wound environment by attenuating the deleterious MMPs, MPO and ROS activities, and the bacterial infection.

The strong antioxidant activity, inhibition of oxidative and deleterious enzymes as well as antimicrobial effect of the generated biopolymer matrices, are potentially beneficial for the wound healing process. Therefore thiolysis of proanthocyanidins and laccase-assisted cross-linking seems to be a promising approach to obtain biopolymeric matrices with enhanced functional properties for wound treatment applications.

6.2 Future remarks

The results of this thesis showed that the new materials are promising candidates for wound dressing materials. Based on the same approaches, new morphologies can be developed in order to adapt the dressing to wound shape and their properties can be further modulated depending on wound requirements.

The materials developed, in a further step, should also be evaluated *ex vivo* through the use of skin/wound models and real wound fluids, and ultimately evaluated *in vivo*. Important parameters such as citotoxicity and cell proliferation should be determined.

Curriculum Vitae

Education

2006 – 2012 **Ph.D. "Development of multifunctional biopolymeric materials for treatment of decubitus ulcers"**
Group of Molecular and Industrial Biotechnology (GBMI)
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2006 **Fronteras de la Química.**
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2005 **Training Course on Valorization and Technology Transfer: Research results valorisation, business plan development, patenting and licensing research and creativity.**
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Other

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Short-term scientific stages

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2005 **KitoZyme (Liège, Belgium)**
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Publications

Research articles

Hossain, K. M. G., González, M. D., **Lozano, G. R**. and Tzanov, T., Multifunctional modification of wool using an enzymatic process in aqueous–organic media. *Journal of biotechnology* 2009, 141, 58-63.

Antonio, F., **Guillem, R**., Sonia, T., Clara, M., Piergiorgio, G., Valeria, C., Gianluca, C., and Tzanov, T. Cross-linked collagen sponges loaded with plant polyphenols with inhibitory activity towards chronic wound enzymes. *Biotechnology Journal* 2011, 6, 1208-1218.

Díaz-González, M., **Rocasalbas, G**., Francesko, A., Touriño, S., et al., Inhibition of deleterious chronic wound enzymes with plant polyphenols. *Biocatalysis and Biotransformation* 2012, 30, 102-110.

Book chapter

A. Francesko, M. Díaz González, **G. R. Lozano**, T. Tzanov. Developments in the processing of chitin, chitosan and bacterial cellulose. *Advances in textile biotechnology* 09/2010: pages 288-311; , ISBN: ISBN 978-1-84569-625-2 (print), ISBN 978-0-85709-023-2 (online)

Research articles submitted

Guillem Rocasalbas, Antonio Francesko, Sonia Touriño, Xavier Fernández-Francos, Georg Guebitz, Tzanko Tzanov. *Laccase-assisted formation of bioactive chitosan/gelatin hydrogel for wound dressings.* (Accepted)

Guillem Rocasalbas, Sonia Touriño, Josep Lluis Torres and Tzanko Tzanov. *A new approach to produce plant antioxidants-loaded chitosan for medical applications*

Work presented at conferences

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Guillem Rocasalbas, María Díaz González, Antonio Francesko, Sonia Touriño, Josep Lluis Torres and Tzanko Tzanov. *"A new approach to produce plant antioxidants-loaded chitosan for medical applications"*
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