



MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES



DEVELOPMENT AND CHARACTERIZATION
OF BIONANOCOMPOSITES FROM ELECTRO
SPUN COLLAGEN/PHBV/CHITOSAN/
HYPERICUM PERFORATUM **EXTRACT FOR**
POTENTIAL WOUND HEALING
APPLICATIONS

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MASTER THESIS

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FEN BİLİMLERİ ENSTİTÜSÜ



PHBV/KOLAJEN/KITOSAN/HYPERICUM
PERFORATUM EKSTRESİ
BIYONANOKOMPOZİTLERİNİN POTANSİYEL YARA
İYİLEŞMESİ UYGULAMALARI İÇİN ELEKTROSPİN
YÖNTEMİ İLE ÜRETİLMESİ VE KARAKTERİZASYONU

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ABSTRACT

DEVELOPMENT AND CHARACTERIZATION OF BIONANOCOMPOSITES FROM ELECTROSPUN COLLAGEN/PHBV/CHITOSAN/HYPERICUM PERFORATUM EXTRACT FOR POTENTIAL WOUND HEALING APPLICATIONS

A wound can be defined as the loss of the integrity of anatomical tissues caused by exposure to various factors such as lacerations, extreme heat or corrosives. The formation of the wound is generally followed by wound healing which is a complex physiological process that involves many different components. It is known that inflammatory response is the starting point of the synthesis of dermal enzymes leading to degradation of extracellular matrix components. Hyaluronidase depolymerized hyaluronic acid, elastin fibers and fibrin are being hydrolyzed by elastase, and collagenase (matrix metalloproteinases-1) breaks the type I collagen and affects the wound healing process.

Since ancient times, various methods have been employed to treat wounds and many of those generally include the covering of the wound area with various materials and plants. In recent years, biopolymers and plant extracts used in combinations as perfect candidates for novel wound dressing materials due to their superior properties when compared with classical wound dressings such as gauze.

In this study collagen and chitosan was extracted from shrimp shell waste material and characterized by FTIR spectra. *Hypericum Perforatum* was macerated with ethanol to obtain plant extract. Electrospinning of the PHBV/Collagen/Chitosan and *Hypericum perforatum* extract was done using 1,1,1,3,3,3,-hexafluoroisopropanol (HFIP) as a solvent. Obtained material characterized by FTIR and SEM. The data obtained from this work suggest that shrimp shell waste is a perfect source to obtain biomaterials such as collagen and chitosan which can be used to produce nanomaterials for biotechnological applications.

July, 2020

Elvy Greg Bienvenu Kebila Moutsinga

ÖZET

PHBV/KOLAJEN/KİTOSAN/HYPERİCUM PERFORATUM EKSTRESİ BİYONANOKOMPOZİTLERİNİN POTANSİYEL YARA İYİLEŞMESİ UYGULAMALARI İÇİN ELEKTROSPİN YÖNTEMİ İLE ÜRETİLMESİ VE KARAKTERİZASYONU

Yara genellikle derinin, kesilmeler, aşırı sıcak veya aşındırıcı maddeler gibi çeşitli faktörlere maruz kalmasının neden olduğu anatomik dokuların bütünlüğünün bozulması olarak tanımlanabilir. Yaranın oluşumunu genellikle birçok farklı bileşeni içeren karmaşık bir fizyolojik süreç olan yara iyileşmesi izler. Enflamatuar tepkinin, hücre dışı matris bileşenlerinin bozulmasına yol açan dermal enzimlerin sentezini hızlandırdığı bilinmektedir. Hyaluronidaz; hyaluronik asidi, elastaz; fibrin ve elastin liflerini ve kolajenaz (matris metaloproteinaz-1) ise özellikle tip I kolajeni hidroliz ederek yara iyileşmesini etkiler.

Eski zamanlardan beri, yaraları tedavi etmek için çeşitli yöntemler kullanılmıştır ve bunların çoğu genellikle yara bölgesinin çeşitli materyaller ve bitkilerle kaplanmasını içerir. Son yıllarda, biyopolimerler ve bitki ekstratlarından oluşan farklı kombinasyonlar, gazlı bez gibi klasik yara örtüleri ile karşılaştırıldığında gösterdikleri üstün özellikler nedeniyle, yeni yara örtü malzemeleri için mükemmel adaylar olarak ortaya çıkmışlardır. Doğal kaynaklardan ve sentez yolu ile elde edilebilen biyopolimerlerin, farmasötik, biyomedikal, terapötik ve yara iyileştirici malzemeler olarak kullanımları önemli bir araştırma alanıdır.

Bu çalışmada, karides kabuğu atıklarından kolajen ve kitosan ekstrakte edilmiş ve FTIR spektrumları ile karakterize edildi. *Hypericum Perforatum*, bitki ekstresi etanol ile maserasyon yöntemi ile elde edildi. PHBV / Kolajen / Kitosan ve *Hypericum perforatum* ekstresi karışımı çözücü olarak 1,1,1,3,3,3, -heksafloroizopropanol (HFIP) kullanılarak gerçekleştirildi ve elde edilen biyomateryal FTIR ve SEM ile karakterize edildi. Bu çalışmanın sonuçları, karides kabuğu atıklarının, biyoteknolojik uygulamalar için kullanılacak kolajen ve kitosan gibi biyopolimerlerin elde edilmesinde mükemmel bir kaynak olduğunu ortaya koymaktadır.

SYMBOLS

% : Percent

C : Concentration

° C : Degree Celsius

Ca²⁺ : Ion Calcium

Cm : Centimeter

μL : Microliter

μm : Micrometer

g : gram

kDa : Kilo Dalton

kV : kilovolt

m : meter

mA : milliamp

M : Molar

mg/mL: milligram per milliliter

Mg²⁺ : Ion magnesium

mM : milli Molar

nm : nanometer

T_g : Glass transition temperature

V : Voltage

v : volume

w : weight

W : Watt

α : Alpha

β : Beta

γ : Gamma

θ : Contact angle

ABBREVIATIONS

AcOH	: Acetic Acid
AMP	: Adenosine mono phosphate
AFM	: Atomic Force Microscope
APS	: Ammonium Persulfate
ASC	: Acid Soluble Collagen
ATR-FT-IR	: Attenuated-Total-Reflection - Infra-red
Bis-acrylamide	: N,N'- methylenebisacrylamide
BSA	: Serum Albumin (Bovine)
CaOH₂	: Calcuim Hydroxide
C-H	: Carbone-Hydrogen
CH₂	: Methyl
CH₃	: Ethyl
DNA	: Deoxyribonucleic Acid
DS	: Dermal Strain
EFG	: Endothelial growth factor
ECM	: Extracellular matrix
FDA	: Food and Drug Administration.
FE-SEM	: Field Emission Scanning Electron Microscopy
FGF	: Fibroblast growth factor
FTIR	: Fourier Transform Infrared Spectroscopy
Gly	: Glycine
hBDs	: Hypergraph Based Data Structure

HEKa	: Keratocynes
HFIP	: Hexafluroisopropanol
HIV	: Human immunodeficiency virus
HP	: <i>Hypericum perforatum</i>
Hyp	: Hydroxyproline
IGF	: Inflammatory growth factor
IL	: interleukine
iNOS	: Oxide nitric synthetase
Ma	: Milliampere
mRNA	: Acid ribonucléic Messenger
N₂OH	: Nitrous Oxide
NaCl	: Sodium Chlorure
Na₂EDTA	: Ethylenediaminetetraacetic Acid, Disoduim Salt
NH₂	: Amide
Nm	: Nanometer
NMR	: Nuclear magnetic resonance
NO	: Oxide nitric
OH	hydroxide
PDGF	: Platelet derived growth factor
PHAs	: Poly hydroxylalkanoates
pH	: Power of Hydrogen
PHB	: Poly hydroxybutyrate
PHBV	: Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

Pro	: Proline
rpm	: Revolution- Per- Minute
SDS	: Sodium Dodecyl Sulfate
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	: Scanning- Electron-Microscope
TCA	: Trichloroacetic Acid
Tris-HCl	: Tris-Chloride Hydroxide
TEMED	: Tetra methyl ethylene di amine
TGF	: Transforming growth factor
Tg	: Glass Transition Temperature
Tm	: Melting Temperature
TNF	: Tumor necrosis factor
USA	: United State of America
USSCs	: Unrestricted Somatic Stem Cells
UV	: Ultraviolet
VEGF	: Vascular endothelial growth factor
W/V	: Weight-per-Volume

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I. INTRODUCTION

I.1 Introduction and Aim

Every day different kind of skin cuts occurs, such as acute or superficial wounds caused by abrasions, cuts and minor burns. These small injuries are generally treated by the patients using first aid dressing materials. These dressings provide a barrier against dirt, bacterial contamination and mechanical factors such as pressure and friction. However this common application of wound dressings at home often generates a dry wound environment (Kuhlmann et al., 2019). The healing of an acute wound in humans and animals is a very complicated event which includes hemostasis/ inflammation phase, proliferation phase, and remodeling phase (Daunton et al., 2012). As a result of the inflammatory response, the expression of dermal enzymes such as hyaluronidase, elastase and collagenase (matrix metalloproteinases-1) was upregulated and during that process hyaluronic acid, elastin and collagen of the extracellular matrix are hydrolyzed. This process might play a major role in the development of skin wounds (Boran et al., 2018).

Since ancient times, different techniques have been used to treat wounds and many of those generally include the use of gauze or various materials to dress and bandage wounds. However, since gauze tend to stick to the wound and may leave fibers and debris, removal of such dressings may cause damage to the healing wound (Daunton et al., 2012). Therefore, more appropriate dressings must have been developed to overcome these shortcomings. Even if gauze has found to be useful in many situations, doctors and health workers must be aware of this material is not optimal and it is not responding to the norms against wound damage anymore when they use it. Woven gauze is detrimental for wound cure because it requires force to remove (Sood et al., 2014).

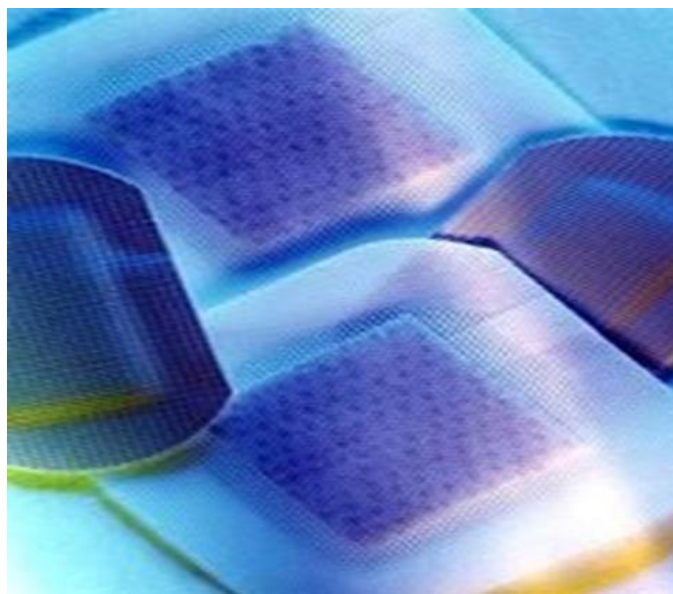


Figure 1.1: An example of novel gauze suggested to promote natural healing (Ball 2003).

In recent years, biopolymers and biochemicals used in combinations as perfect candidates for novel wound dressing materials due to their superior properties such as the ability to maintain a non-traumatic, non-adherent, non-toxic and moist wound environment and favoring antibiotic and gas permeability while preventing maceration of the surrounding tissue, (Martineau and Shek 2006). Several kinds of biopolymer wound dressings within the function to preserve wound hydration and to optimize skin regeneration, protect against bacterial infection and avoiding disruption of the wound base have been found in the market (Patel et al., 2019) In Figure 1.1 an example of a novel gauze was displayed. Polymers are large molecules that made up of repeating building blocks called as monomers (Rao et al., 2014). In Figure 1.2, a polymer synthesis from a monomer is shown.

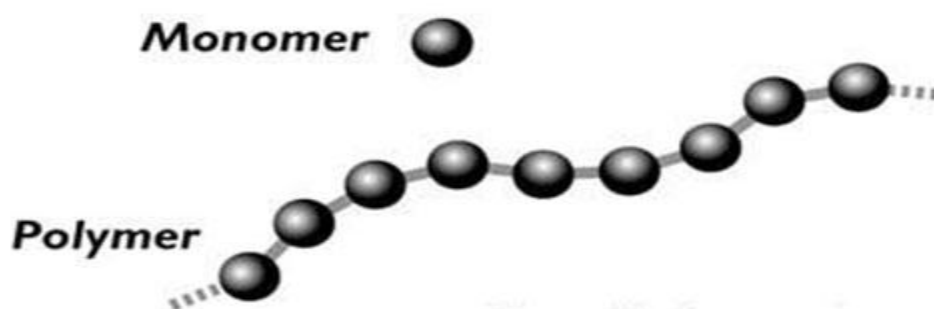


Figure 1.2: Explanation of polymers synthesis from monomers (<https://galaxact.wordpress.com/2015/03/17/classification-of-polymers-and-monomers>).

Biopolymers are natural polymers formed under normal conditions during organisms' growth cycles. Researchers have been investigated in many fields the different possibilities to use biopolymers from natural and synthetic sources for many years and nowadays it has been playing a major role in pharmaceutical, biomedical and therapeutically application. Due to their biological properties favoring fast wound healing, biopolymers are exploited as wound dressings materials (Smith and Morris, 2016). Biodegradable and biocompatible biopolymer structures containing cells or signal molecules are being developed in tissue engineering science. These clinical approaches are already used for tissue transplantation and reconstruction (Sezer et al., 2011). Collagen, polyhydroxyalkanoates and chitosan are among the most frequently used biomaterials for the development of wound dressings and wound healing materials.

Collagen is one of the most common protein found in animal kingdom and more than thirty forms of collagen have been obtained from different species (Friess 1998). Throughout organ development, after skin injury collagens contribute to the local storage and delivery of growth factors and cytokines. Because of their role in tissue repair and wound healing and collagen widely used in wound dressings (Gelse et al., 2003). A family of biodegradable polyesters, polyhydroxyalkanoates (PHAs) are produced by an extensive variety of natural or recombinant bacterial organisms for carbon and energy storage (Li et al., 2016). Fibers of PHAs have been blended with a variety of polymers in laboratory and have demonstrated improved mechanical and biological properties and therefore they can be used to reinforce the stability and efficacy of the wound dressing materials (Li et al., 2016). Amongst the PHAs, poly (3- hydroxybutyrate-3-hydroxyvalerate) (PHBV) is one of the most commonly used polyesters in the field of biotechnology (Tan et al., 2014).

Chitosan (D-glucosamine (β 1 \rightarrow 4) N-acetyl-D-glucosamine) is a linear polysaccharide and produced by the deacetylation of chitin (Krajewska, 2004). Because of its various useful properties such as homeostatic effect, chitosan is being used in the treatment of wounds and skin burns and it has been thought that chitosan might accelerate the formation of fibroblasts which is a major element of proliferation phase in injury and increases early reactions related to healing (Paul and Sharma, 2004).

Hypericum perforatum L. (known as Sarı Kantaron in Turkey), is a member of the genus *Hypericum* family, and it is widely distributed in Europe, West Asia, and North Africa. The extracts of *Hypericum perforatum L.* contains naphthodianthrones, phloroglucinols, flavonoids,

bioflavonoids, and phenylpropanoids which promote the wound healing process and have antifungal, anti-inflammatory, antimycobacterial, and antiviral activities (Yadollah-Damavandi et al., 2015).

Compared to other nanofiber fabrication methods, electrospinning provides various advantages in terms of material selection and control. Electrospinning technique allows to have the ultra-fine fibers with desirable characteristics like a large surface area, a good volume ratio, porosity, and other surface functionalizations, therefore electrospun materials are selected to be used in various biomedical applications including wound engineering such as wound dressing's material (Khan et al., 2018).

In this study, to find sustainable new wound-healing material, we extracted collagen and chitosan from shrimp shell waste. Ethanol extracts of *Hypericum perforatum* was obtained by maceration. Collagen, chitosan, *Hypericum perforatum* extracts and PHBV then electrospun to produce bionanomaterial using 1,1,1,3,3,3-hexafluoro-2-isopropanol (HIFP) as a solvent. The nanofiber mats was characterized via Fourier transform infrared spectroscopy (FTIR), and SEM analysis. Activity of *Hypericum perforatum* extract and biomaterial on collagenase activity was also studied.

1.1 General Information

1.2 Wound healing definition and process

A wound can be defined as the disruption of the integrity of anatomical tissues caused by exposure to various factors such as lacerations, extreme heat, or corrosives (Kio and Kubo 2019). The formation of the wound is generally followed by wound healing which is a multi-factorial, complex physiological process that involves many different cell types, the extracellular matrix, and mediators, such as neuropeptides, growth factors, and cytokines (Kio and Kubo 2019). The mechanism of wound healing which is a skin repair process has been organized in three sequential and overlapping steps: The inflammatory phase, which includes cutaneous neurogenic inflammation and hemostasis (Figure 1.3). The proliferative phase, responsible for the increase of several organs and the remodeling phase, involving the formation of the scars; these events start very early in the first seconds after injury (Cañedo-Dorantes et al., 2019).

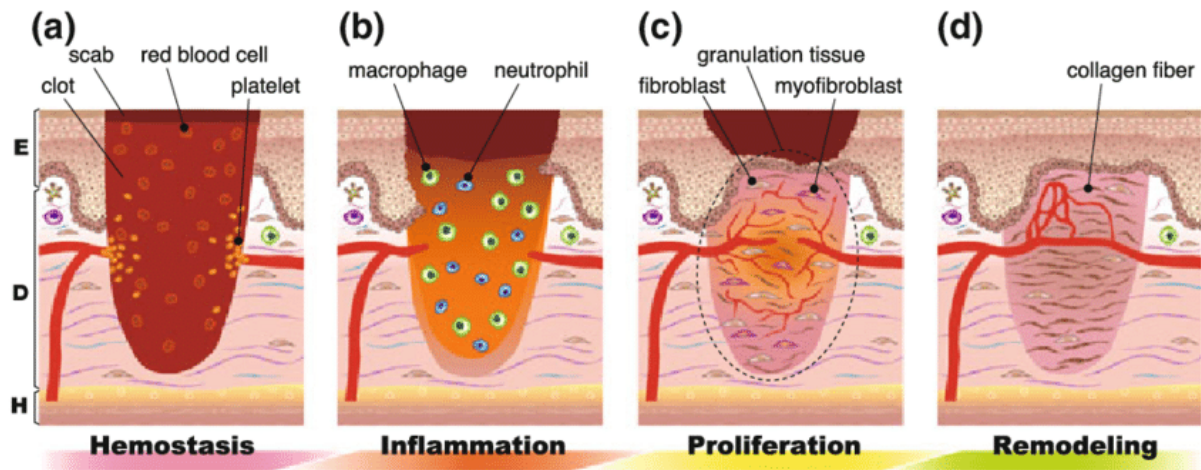


Figure 1.3: Wound healing process (Kawasumi et al., 2012)

Hemostasis phase

Hemostasis is the first step of a wound healing process. Early after a skin injury, the wound bed is being full of capillary blood, and this event is followed by a mechanism to reduce continuous blood loss. Humoral and other components and cells, such as fibrinogen, platelets, and stem cells provide signals that contribute to the earliest phases of wound healing (Kawasumi et al., 2012). To prevent exsanguination at the site, vasoconstriction occurs and platelets become activated when exposed to extravascular collagen (such as type I collagen). After come in contact with collagen, soluble mediators such as growth factors and cAMP and adhesive proteins were released from thrombocytes (Schultz et al., 2011). The vascular constriction and fibrin clot formation surrounding the wound tissue release pro-inflammatory cytokines and growth factors such as transforming, fibroblast, epidermal and platelet-derived growth factor (TGF)- β and α , (FGF),(EFG) and (PDGF). Once the blood flow has been contained, inflammatory cells such as macrophages, neutrophils and lymphocytes move into the wound and started the inflammatory phase (Broughton et al., 2006; Guo and DiPietro, 2010; Campos et al., 2008). Meanwhile, VEGF, TGF- α , and bFGF activate endothelial cells and initiate angiogenesis and in other parts, fibroblasts are also being activated and recruited by PDGF to migrate to the wound site and the production of collagen and glycosaminoglycans starts (Schultz et al., 2011).

Inflammatory phase

The second stage of wound healing starts with components of cells (platelets, erythrocytes, and fibrin), mixed with extravasated serum proteins, tissues, and foreign material introduce when the injury happened. The release of signaling molecules coming from the wound site throughout

hemostasis gives information about the initiation of the inflammatory phase. In that part, platelets are being released by chemoattractant molecules, also vasodilation and vascular permeability increased, subsequently raising leukocyte recruitment (Stroncek and Reichert, 2008). In this phase, neutrophils and macrophages are the first cells to appear at the injury place and then accumulate and facilitate phagocytosis of bacteria from damaged tissue (Wang et al., 2018). Because they cleanse the wound infection, debris and mediators solution released such as proinflammatory cytokines (including IL-1, IL-6, IL-8, and TNF- α), and growth factors (such as PDGF, TGF- β , TGF- α , IGF-1, and FGF), those cells are the main key cells during the inflammatory phase and play a great role during this step (Schultz et al., 2011). The maintenance of the integrity of this phase is being done by the lesioned blood vessels contract and the leaked blood coagulates. The aggregation of thrombocytes and platelets in a fibrin network is what this coagulation consists of and aims to rely on the action of specific factors through the activation and aggregation of these cells. In addition to reestablishing homeostasis, the fibrin network forms a barrier against the invasion of microorganisms, organisms that are the necessary temporary matrix for cell migration (Gonzales et al., 2016).

The proliferative phase

The establishment of a new matrix of collagen fiber, proteoglycans, and fibronectin to restore the structure and function of the destroyed tissue like the fibrin matrix still represents the main stages of the proliferation phase (Schultz et al., 2011). One of the other essential events in wound healing is angiogenesis, which is more precisely the growth of new capillaries to replace previously damaged vessels and restore circulation. The healing phase is also governed by other important events such as the formation of new granulation tissue and epithelialization. Several cells such as like fibroblast cells are the keys to the proliferative phase during healing (Schultz et al., 2011). Myofibroblasts promote wound contraction by grabbing the edges of the wound and bringing them together while using a mechanism similar to that of smooth muscle cells (<https://www.woundsource.com/blog/four-stages-wound-healing>).

Remodeling phase

Two weeks after the appearance of the lesions, the third phase of healing begins and follows the stage of remodeling and resynthesizes of the extracellular matrix (Gonzales et al., 2016). During this phase of remodeling and maturation, the excess of myofibroblast cells and then resident extracellular matrix disappears by subsequent elimination of senescent cells by the activity of the innate immune system (Wlaschek et al., 2019). Once the surface of the lesion is

completely covered with a monolayer of keratinocytes, the closure of the wound begins to appear, the type III collagen undergoes degradation then the synthesis of type I collagen increases. Throughout cell reshaping, the concentration of hyaluronic acid is reduced and fibronectin is transformed into other products by specific cells and plasma metalloproteinase. At this final level of healing of the lesion, there is the appearance of an attempt to recover normal tissue structure and a gradual reshaping of the granulation tissue, thus forming scar tissue (Gonzales et al., 2016).

1.3 Different types of wound

Wound healing is a normal biological process of human metabolism, so there are several types of sores that appear after a tear in the skin (Kio and Kubo 2019). The wounds can be of various orders, open, closed or ulcerated and depending on whether they are of different shapes and characteristics, the body tissues are arranged in a variant manner. They are exposed at the base in open wounds and in the case of closed wounds, damage occurs without exposing them. several internal or external causes can be at the origin of injuries, such as the penetration of objects, blunt trauma or immune, metabolic and neurological etiologies. The most common types of sores are bruising, seroma, pressure, diabetic foot and ulcers (<http://www.jobst-usa.com/healthy-living/wound-care/wound-types/>). In Figure 1.4, different types of wounds are illustrated.

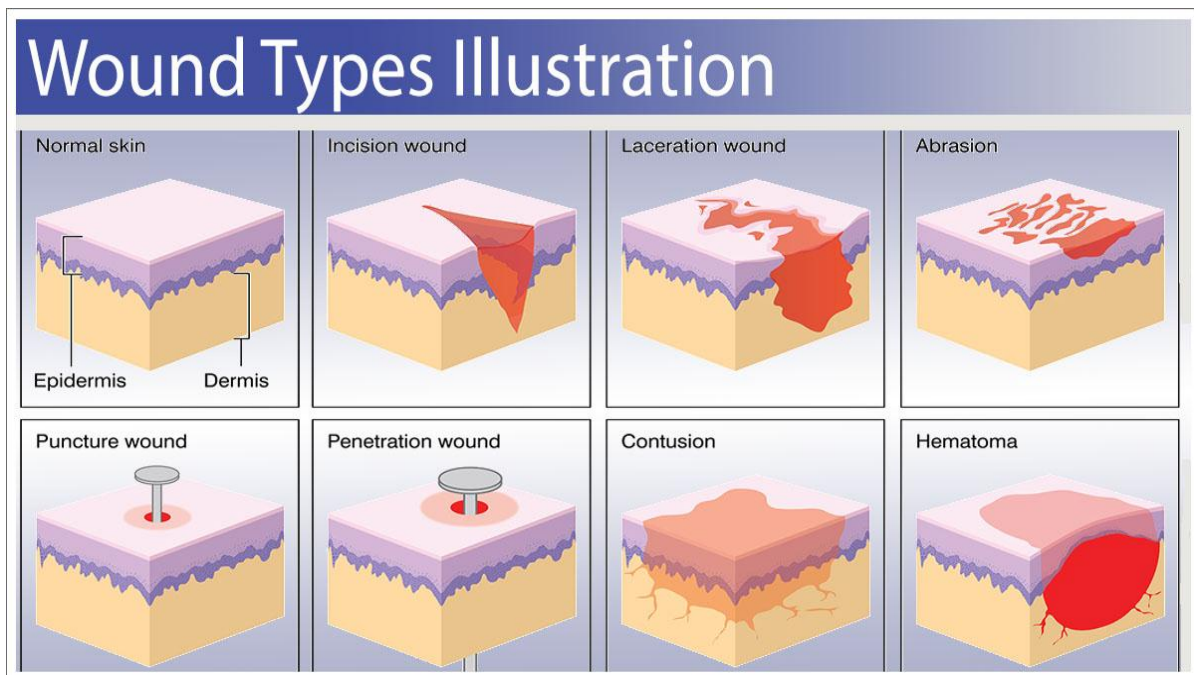


Figure 1.4: Different types of wound (<https://www.biodermis.com/understanding-different-types-of-wounds>)

1.4 Four tissue types of wound healing

Class I/ clean wounds - These are surgical wounds, uninfected mainly closed and, if necessary, drained in which no organism causing any inflammation is found and or no respiratory tract, food, or strain infected did not return (Speicher et al., 2014).

Class II / Clean contaminated wounds - These are wounds of a surgical nature in which, under controlled conditions or without excessive contamination of the hutches with respiratory, food, genital or urinary characteristics are introduced (Chhabra et al., 2017).

Class III / Contaminated wounds - These are wounds coming from surgical intervention or not. They are open, more or less fresh and are the result of major interruptions in the sterile technique or a brutal discharge from the gastrointestinal tract and incisions in which acute non-purulent inflammation is encountered through infectious agents.

Class IV / Dirty Infected wound - They are old traumatic with retained or devitalized tissue and or perforated viscera and which involve an existing internal clinical infection or else due to the presence of an organism causing a post-operative infection present before the intervention surgical (Speicher et al.,2014).

1.5 Microorganisms (bacteria) involved in wound healing activity

After every injury, wounds are simultaneously colonized and then invaded by aerobic and anaerobic microorganisms, precisely pathogenic microorganisms that mainly come from mucous surfaces and the environment in which we live. These organisms are generally gram-positive and gram-negative bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *E. coli*, *S. aureus* and *P. aeruginosa* (Gomes et al., 2017). In Figure 1.5, prevalence of the common bacteria isolated from healing and non-healing wounds were shown. Different bacterial species have been also observed on the chronic wound after an injury such as *Staphylococcus epidermis* and those kind has been causing significant deep tissue damage (Traci 2017; Savitskaya et al.,2019). The role of these microorganisms are pathogenic, but the importance they play in the wound healing process has been debated for many years. While some microbial pathology experts consider microbial density to be essential for assessing and predicting wound healing and infection, others claim that in arriving at this assessment the presence of specific microorganisms is more important than others (Bowler et al., 2001).

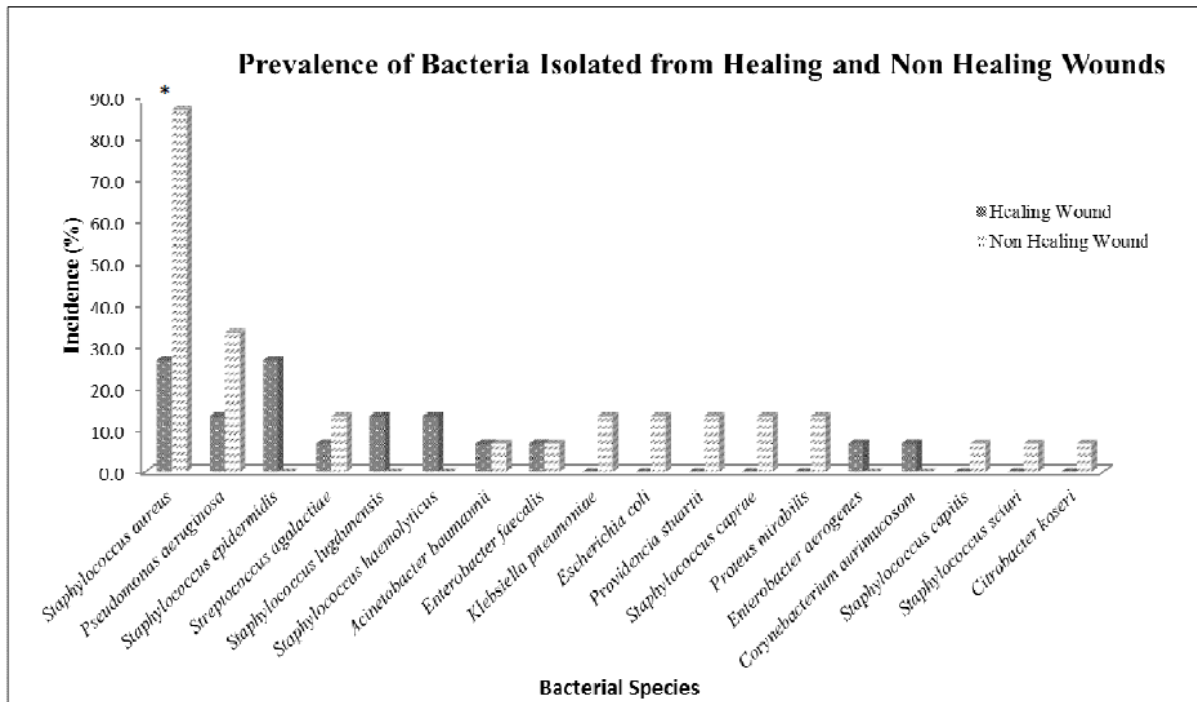


Figure 1. Prevalence of bacteria isolated from 15 healing and 15 non-healing wounds.
 *Significant difference in prevalence in healing and non-healing wounds at $p < 0.05$

Figure 1.5: Bacteria isolated from healing and non-healing wounds (Wong et al., 2013).

1.6 Biomolecules found on wound healing activity

After the beginning of wound healing activity, some cell types start being produced and their effect is very important (Ganapathy et al., 2012). Several molecules of the ECM play an essential role in wound healing, such as structural proteins and more particularly collagen and elastin, as well as adhesive glycoproteins, glycosaminoglycans/proteoglycans and the category of matrix proteins (Traci 2017). Endogenous human collagen which promotes the manufacture of extracellular proteins constituting the ECM and the reconstruction of the structure. Also several endogenous antimicrobial peptides (AMP) are susceptible to behave like healing peptides, thus displaying a dual antimicrobial and regenerative property or generator of new tissues such as β -defensins (hBDs), cathelicidin LL-37 and dermcidin. HaCaT and HEKa keratinocytes, temporin B and non-antimicrobial thrombins peptides that promote skin repair and play an important role in blood clotting (Gomes et al., 2017)

Other cells have been identified, such as platelets, neutrophils, macrophages, T-lymphocytes, and fibroblasts primarily contribute to the process (Mohd et al., 2012). Platelet activation release growth factors such as platelet-derived growth factor (PDGF), TGF- α and TGF- β , FGFs, TNF- α and mediators that cause many biological metabolisms such as stimulating or inhibiting fibroblasts for TGF and FGF, the stimulation of angiogenesis by macrophages due

to TNF (Ellis et al., 2018). In the same order, there is an aggregation and then activation of the subendothelial collagen, gradually leading to the formation of a hemostatic plug due to the release of cytokines and growth factors. Also, neutrophils defend themselves against infectious agents of wounds which are mostly proteases, microbial proteins, and phagocytizing pathogenic agents and kill them by the release of reactive species of the oxygen (Ellis et al., 2018). The cells which are involved wound healing are listed in Table 1.1. Macrophages, on the other hand, essentially perform the phagocytosis of muscle debris and residues, as well as the production of cytokines and pro-angiogenic, inflammatory, fibrogenic, and free radical factors (Gonzales et al., 2016). While the lymphocytes, which are very important cells responsible for the production of IL-2 cells help in the recruitment of fibroblasts and are the last to intervene in wound healing.

Name	Abbreviation	Class	Produced by	Action
Interleukins 1, 6, 8	IL1, IL6, IL8	Cytokines	Macrophages, keratinocytes	Proinflammatory; recruit fibroblasts and keratinocytes
Interleukin 2	IL2	Cytokine	T lymphocytes	Recruits fibroblasts
Interleukin 4	IL4	Cytokine	T lymphocytes	Inhibits TNF, IL1, IL6, inhibits fibroblast proliferation
Tumor necrosis factor alpha	TNF- α	Cytokine	Macrophages	Proinflammatory; helps collagen synthesis
Epidermal growth factor	EGF	Growth factor	Platelets, macrophages, keratinocytes	Promotes keratinocyte and fibroblast proliferation, keratinocyte migration, and granulation tissue formation
Fibroblast growth factors acidic and basic	FGF-a and b	Growth factors	Endothelial cells, fibroblasts, macrophages, T lymphocytes	Cause angiogenesis, fibroblast chemotaxis and proliferation
Keratinocyte growth factors 1 and 2	KGF	Growth factors	Fibroblasts	Stimulate keratinocyte division and differentiation
Platelet derived growth factors (PDGF exists in several forms: AA, BB, AB, others)	PDGF	Growth factor	Platelets, macrophages; also fibroblasts, endothelial cells	Cause neutrophil and fibroblast chemotaxis; fibroblast proliferation, and synthesis of matrix proteins, metalloproteinases, stimulates angiogenesis
Transforming growth factors (alpha and beta)	TGF- α,β	Growth factors	Platelets, macrophages, fibroblasts, keratinocytes, T lymphocytes	Cause fibroblast and keratinocyte chemotaxis, angiogenesis; upregulates TIMP; inhibits production of MMPs and keratinocyte proliferation, induces TGF β production
Vascular endothelial growth factors (a family of peptides)	VEGF	Growth factors	Endothelial cells, keratinocytes, platelets, macrophages, fibroblasts	Cause angiogenesis (mitogenic for endothelial cells). Expression increased in the presence of hypoxia
Tissue inhibitor of metalloproteinase	TIMP	Enzyme	Most mesenchymal cells	Inhibits MMPs
Matrix metalloproteinases	MMPs	Enzymes	Monocytes, macrophages, endothelial cells	Degrade the extracellular matrix

Table 1.1: Cells which are involved in wound healing events (Mohd et al., 2012).

1.7 Collagen and definition

Collagen is the main component of the scaffold, containing various amino acids. Collagen is the major constituent of extracellular matrix (ECM) which is formed with crosslinked monomers building fibril and found in connective tissues, mainly the cartilage, animal skin, veins, bone and tendons (Muyonga et al., 2004; Mocan et al., 2011). It is mainly used in many industries including food, pharmaceutical, and cosmetic industries (Song et al., 2014, Kim et al., 2020). Collagen is the main constituent giving firmty and stability to organs and tissues and

its ability to stretch and regains its original shape by elastic fibers. Elastin is the central core of elastic fibers (Krafts 2010). Collagen's three-dimensional triple helix structure is given below in Figure 1.6.

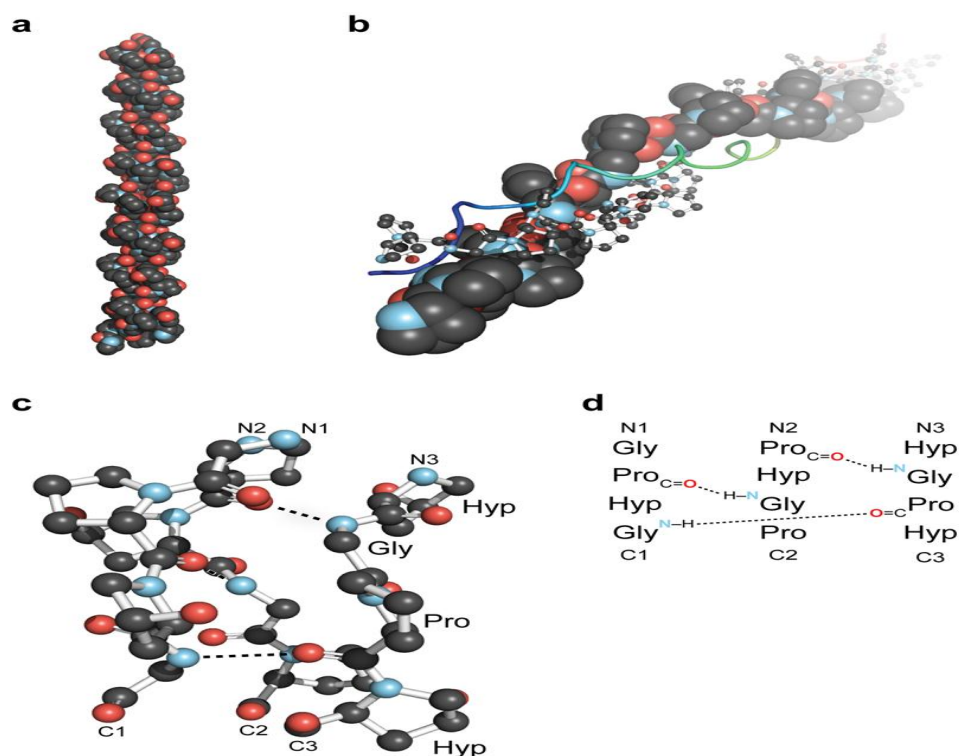


Figure 1.6: Collagen structure (a) Crystal triple helix. (b) 3D image of a turn (c and d) hydrogen bonds in segment b (Shoulders and Raines, 2009).

Collagen's architecture affects its functionality, which makes sense to understand how the structure and hierarchical order of scaffolding proteins are represented in the space, like the electrospun collagen fibers for example. Glycine-X-Y is the representation of the primary arrangement of the α -chains of collagen molecules, composed of repetitive subunits of amino acid sequence (Shoulders and Raines, 2009). Possessing a representation of the alpha helix which twists on the left, three of these alpha-helix subunits are wound together on itself with a twist to the right when describing a triple helix or tropocollagen molecule. In this structure X and Y impose the choice of specific amino acids, due to the tight conformation, as soon as the helical force makes each third residue imperatively a glycine. Therefore, the chemical structure of collagen fiber is revealed composed of amino acids proline (28%) and 4-hydroxyproline (38%), representing either X for one or Y for the other depending on whether it is (Sizeland et al., 2018). The representation of the chemical structure of collagen fiber is corroborated by Emil Fischer who stipulates a repetition of Xaa-Yaa-Gly referring to a triplet and where Xaa and Yaa

represent individual amino acids such as -4-hydroxyproline (Hyp) and proline (Pro) as mentioned above. The tight compression of the strands in triple helix of tropocollagen is greatly favored by the repetition of the Gly molecule. The resulting molecule is therefore as it follows Pro,Hyp, and Gly and is the frequent representation of triplet in the structure of collagen (Chattopadhyay and Raines, 2014).

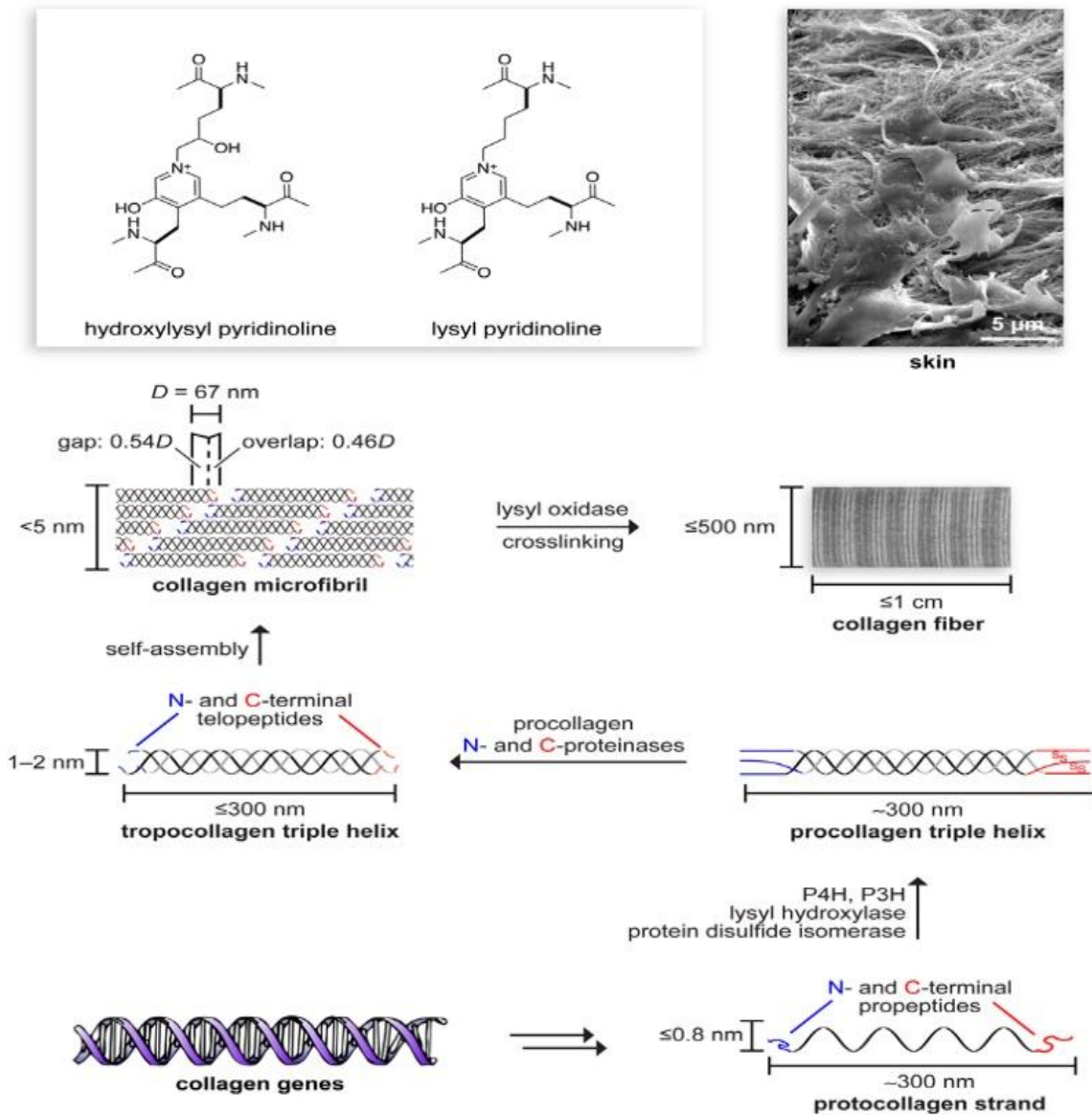


Figure 1.7: Biosynthesis of natural and synthetic collagen triple helices and fibrils (collagen type I) (Shoulders and Raines, 2009).

1.7.1 Collagen Types

There are many types of collagen, but only a few of them are used in the production of collagen-containing biomaterials. Collagen is used in many industries, such as leather, cosmetics, food and has applications in tissue engineering and pharmaceutical industry (Liu et al., 2012). Almost 1/3 of the whole proteins in humans is collagen and it makes up 3/4 of the dry weight of skin. Today, researchers have identified 29 types of collagen (Chattopadhyay and Raines, 2014).

Collagen is a triple, right-handed helix containing three α -chains, collagen types are ordered according to their characteristics while type II, III, VII, VIII, and X collagens possess three identical chains of I, IV, V, VI, IX, and XI type collagen molecules have two more distinct chains. In Figure 1.7 biosynthesis of natural and synthetic collagen triple helices and fibrils (collagen type I) and in Figure 1.8 structures of type I, III, IV, V, and VI collagens were shown.

Helical triple forms the quaternary structure of collagen and contains strong and weak bonds and forming fibrils and these bonds favor its stability (Brinckmann et al., 2005).

In a collagen molecule, 18 amino acids are present for each turn and forms a left-handed extended helix each having three α -chains (Gelse et al., 2003).

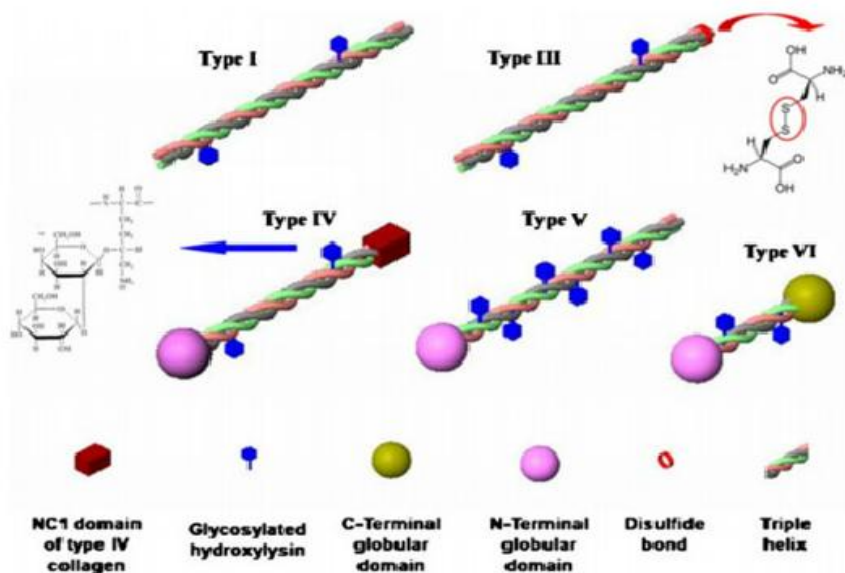


Figure 1.8: Different structures of collagen type I, III, IV, V, and VI (Gorgieva and Kokol., 2011).

1.7.2 Collagen Sources

Main constituent of fibrous proteins is collagen is mainly present in the connective tissue of higher animals; (Gómez-Guillén et al., 2011). Collagen is the main constituent in tendons, skin, cartilage and bones. In Table 1.2 different types of collagen are listed. Because the risk of mad cow disease and ban on pork in Muslim countries, the incident of obtaining collagen has been directed to different animals such as fishes and birds (Schmidt et al., 2016). Eggs have also been accepted as a good collagen source (Mohammadi et al., 2016).

Type	Peptide Chains	Molecular Composition	Occurrence
I	$\alpha 1, \alpha 2$	$[\alpha 1(I)]_2 \alpha 2(I)$	Skin, tendon, bone, muscle (epimysium)
II	$\alpha 1$	$[\alpha 1(II)]_3$	Intervertebral disc, cartilage
III	$\alpha 1$	$[\alpha 1(II)]_3$	Fetal skin, cardiovascular vessel, uterus, synovial membranes, inner organ, muscle (perimysium)
IV	$\alpha 1, \alpha 2$	$[\alpha 1(IV)]_3$	Basement membrane, kidney glomeruli, lens capsule, glomeruli
V	AA, $\alpha B, \alpha C$	$[\alpha B]_2 \alpha A$ or $(\alpha B)_3$ + $(\alpha A)_3$ or $(\alpha C)_3$	Placental membrane, cardiovascular system, lung, muscle (endomysium)

Table 1.2: Types of Collagen and part of the body where it is found

1.7.3 Biomedical Applications of Collagen

The application of biopolymers as scaffolds has always been related to repairing lost or damaged tissue and organs. Collagen-based materials on the market are successfully used for skin repair. Animal or and recombinant collagens, mainly type I collagen, are nowadays substantially used for, esthetic surgery, tissue engineering and drug delivery. United States Food and Drug Administration (FDA) has approved Collagraft®, Angiotech Pharmaceuticals as a synthetic bone graft replacement by the) is a blend of collagen, hydroxyapatite, and tricalcium phosphate) (Song et al., 2018). Collagen sponges are also used as an effective scaffold for the biomedical applications. For example, a spongy collagen matrix which is composed of oxidized cellulose; Promogran® (Systagenix) is marketed for of diabetic and ulcer wounds treatment, is now in the wound healing market (Vin et al., 2002).

Interestingly spongy nature such as sponges of type I collagen have also been used to tissue replacement studies in rabbit patellae tendons. the repaired tendon tissue acquired the

mechanical properties of patellae tendon by 75% (Juncosa-Melvin et al., 2006). Many studies report that collagen is successfully used as a scaffold in bladder and nerve engineering. In addition to these, a three dimensional collagen graft suture-free, DuraGen® (Integra LifeSciences) was approved by the FDA and has been introduced into the market (Narotam et al., 2004). Collagen implants have often been used as a means for the delivery of cultured keratinocytes and antibiotics and other drugs for the treatment of skin burns. In addition to this technology, various journals report that by immobilizing curative enzymes or regulating the administration of drugs to patients and collagen containing certain chemical compounds such as acetyl, succinate or other biomaterials have been used in the biomedical applications (Chattopadhyay and Raines 2014).

1.7.4 Anti-inflammatory activities of collagen

Usage of many types of collagen have been increased in many fields such as skin and certain organs due to their potential anti-inflammatory, anti-microbial, proliferative and antioxidant activity profiles (Offegenden et al.,2018). Collagen peptides which are hydrolyzed into di- and tripeptides by collagenase action have been shown to display inhibitory activity on lipoxygenase and nitric oxide (NO) which are responsible for several allergic and inflammatory reactions. This inhibitory activity may be due to the –OH and –NH₂ groups of collagen peptides which are interacting with nitric oxide radical and lipoxygenase (Hakuta et al., 2017).

1.7.5 Wound healing activity of collagen

The development of a new tissue on skin injury is a very complicated task process that includes many phases such as inflammation, angiogenesis, formation of granulation tissue, re-epithelization, and rebuilding of ECM. The collagen synthesized by the fibroblasts is directly associated to the strength and entirety of the newly build blood veins (Bolla et al.,2019). Connective tissues cannot heal by regeneration and repair process, mostly depend on the building of a scar tissue containing collagen (Berry et al, 1998) Especially Type I collagen, contributes to restoration, constancy, strength and function of the tissue. In case of an injury and a subsequent repair process begins, collagen is also very critical in in the control of inflammatory response which is related to phases; cellular mitogenesis, differentiation and migration. Fibroblasts form fibrous tissue which emigrate to the wounded area, proliferate and synthesize collagen with the cooperative action of plenty of cytokines and growth factors (Krafts 2010). Finally, fibroblasts turn into myofibroblasts which increase collagen deposition and trigger wound contraction (Felician et al., 2019).

1.7.6 Collagen extraction and isolation methods

Collagen is mostly extracted by hydrolysis which may be chemical and enzymatic (Zavareze et al., 2009). Animals like porcine or fish skin, bones, and others are usually used for collagen extraction. Chemical hydrolysis is frequently used in industry, but when improved functional products with enhanced nutritional values are demanded enzymes are more promising and biological processes are preferred (Schmidt, et al., 2016). For extraction of collagen, it is vital to delete many covalent intra- and intermolecular cross-links contributing the process and making it quite complex. Before an alkaline or acid hydrolysis, a pretreatment is necessary for removing non-collagenous substances and obtaining high yield, based on the origin of the collagen. Collagen present in connective tissues dissolves very slowly. That's why, before the extraction, chemical process is required to break the cross links in collagen molecule. In the acidic extraction, the raw material is soaked in acidic solution until the solution diffused into the material. Temperature controlled acidic solution is ingested and the material, swells two or three times to its initial volume and the inter-and intra-molecular non covalent bonds are mostly broken (Ledward 2000). The acidic process is relevant for gentle raw materials, such as porcine and fish skins (Almeida et al., 2012). The alkaline process includes mostly sodium hydroxide (NaOH) and $\text{Ca}(\text{OH})_2$. It may take a few days or may be a few weeks and used for thick materials such as bovine ossein.

Chemical hydrolysis

Neutral salt solutions as sodium chloride (NaCl), Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride), phosphate and citrate salts are used for the extraction of salt soluble collagen. Controlling the salt concentration is critical and the method is limited of, since the structure collagen molecules is mostly composed of cross links (Yang and Shu, 2014). Mild organic acids or strong inorganic acids may be used. But, organic acids are proven to be more efficient than inorganic acids. Collagen is highly soluble in organic acids which can break inter-cross-links (Liu et al., 2015). So, acetic acid, are generally used for collagen extraction. Then filtering is performed and the filtrate is precipitated and then dialyzed against water.

Enzymatic hydrolysis

0.5 M acetic acid solution containing proteases such as pepsin is added to the acidic hydrolysate. After continuously stirring the mixture for 48 hours, at a temperature of 4°C, the

mixture is filtered (Li et al., 2013). The conditions in the rest of the process is same for obtaining acid soluble collagen then the hydrolysate is filtrated.

1.8 Chitosan and definition

Chitosan is a polysaccharide which is obtained by the deacetylation of chitin in the presence of certain chemicals such as NaOH. It is composed of -glucosamines linked to the β - (1-4) of N-acetyl-glucosamines (Krajewska 2004). In Figure 1.9, chemical synthesis of chitosan is described. Crustacean exoskeletons, fungi cell walls and bacteria are the general sources for chitosan extraction and several processes are used (Rinaudo 2006). This polymer is found naturally and abundantly and more than 1000 tons are produced each year from different sources. (Cui and Nair, 2010).

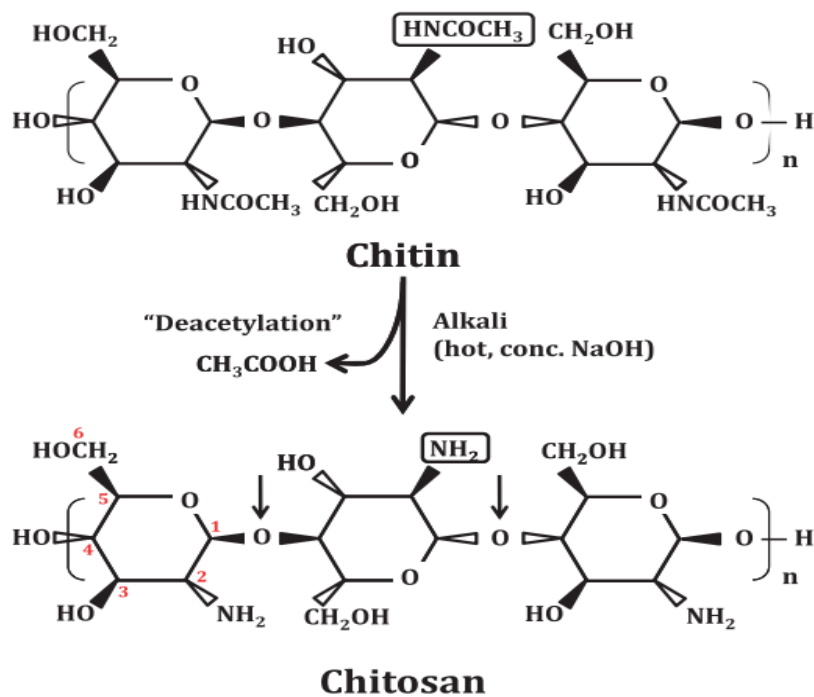


Figure 1.9: Chemical synthesis of chitosan (Raafat and Sahl, 2009)

N-acetyl-D-glucosamine is found in three polymorphic arrangements as α , β , γ , and many products derived from chitin have different properties. The most abundant form is α -chitin, its configuration is anti-parallel, crystalline, highly ordered, and has strong H-bonds which have characteristics like rigid, intractable and soluble. The β -chitin form is found in diatom species and has a parallel configuration. Its H-bonds are weak and unstable and soluble in water. The γ -form is a mixture of α , β and has intermediate properties.

1.8.1 Properties of Chitosan

Due to the nature of its cationic polysaccharide chain chitosan, may be neutral or acid. In an acidic solution chitosan has many distinctive properties (Cheung al., 2015). One of its features is the ability to produce complexes with natural and synthetic biopolymers which are electrostatic or multilayers. Being non-toxic, biocompatible, low allergenic and biodegradabile material chitosan is used in various applications. Biological properties like antimicrobial, antifungal, antitumoral and antioxidant activities are given in Table 1.3 (Cheung et al., 2015).

1.8.2 Chitosan isolation methods

Crustacean exoskeleton is the major source for the commercial chitosan. The production from the biological materials generally involves demineralization, deproteinization, decoloration, and deacetylation basic steps: Typical flow chart for chitosan manufacturing is given below in Figure 1.10:

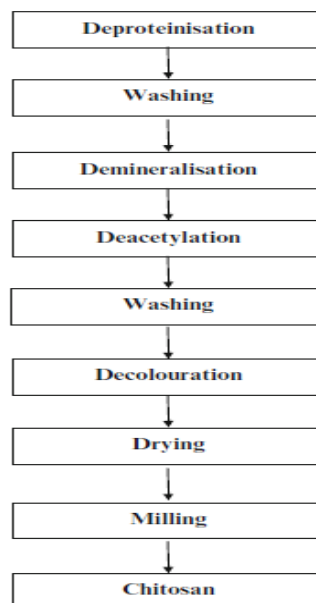


Figure 1.10: Flow chart for the chitosan manufacture (Ramawat and Merillon, 2015)

Chitosan samples produced are characterized by methods using nuclear magnetic resonance (NMR), Fourier transforms infrared (FTIR) spectras and scanning electronic microscopy (SEM) images (Amer and Ibrahin, 2019).

Table 1.3: Physical and chemical characteristics of chitosan and determination methods (Cheung et al., 2015)

Physical and chemical features	Methods used
Crystallinity	X-ray
Deacetylation degree	UV and IR spectrophotometry; NMR (¹ H-NMR and ¹³ C-NMR); conductometric and potentiometric titration; differential scanning calorimetry
Molecular weight	Viscometry; gel chromatography; light scattering; HPLC X-ray diffraction, mass spectrometer

1.8.3 Biomedical applications of chitosan

Chitosan is a biocompatible, biodegradable, non-toxic biopolymer having polyelectrolyte character. It can form gel easily. In addition to these features has antimicrobial and antitumor properties. It has vast applications in medicine. It is used in drug delivery systems, artificial skin, hemostatic agents, and hemodialysis membranes, Chitosan forms gels in slightly acidic solutions so it displays anti acid and antiulcer activities. When intravenously injected, chitin and chitosan oligosaccharides, activate macrophages and show antitumor activity, Chitin and chitosan have been used as a support for immobilization of enzymes used in the synthesis of organic compounds, wine industry (Ravikumar 2000). Chitosan is also used in the design of biosensors for measuring environmental pollutants and in artificial organs for metabolite control (Krajewska 2004). Interest of researchers, pharmaceutical and biomedical companies is substantially increasing for chitosan and chitin because it may be used for drug carrying, water treatment and wound healing, and in tissue engineering as scaffolds and is a very popular topic of many publications. Chitosan as a nanocarrier is used in saquinavir which is an anti-HIV-1 drug with outstanding drug loading capacity and a considerable T-cell targeting performance (Cheung et al., 2015). N-methacryloyl chitosan, produced by N-acylation reaction, attains very good properties like solubility, injectability and is able to make UV crosslinks. It stimulates fast and profitable, cheap construction of cell-immobilized microgels containing special amino groups as building blocks for tissue engineering (Cheung et al., 2015). Scaffolds containing chitosan glass nanoparticles having the shape memory feature of chitosan have many applications in bone mineralization (Correia et al., 2015).

1.8.4 Anti-inflammatory and anti-bacterial activities of chitosan

Chitosan is antimicrobial against a broad-spectrum of Gram-positive and Gram-negative bacteria, yeasts and fungi (Venugopal 2011). Its antibacterial activity is attributed to its cationic nature and low molecular weight. While bulky chitosan can bind to negatively charged bacterial cell wall constituents and form a waterproof layer and as a result permeability of the cell changes and low-molecular-weight chitosan can pass through the bacterial cell wall and bind with DNA. By this way, DNA transcription then the mRNA synthesis are inhibited. Chitosan displays stronger activity on gram-negative bacteria when compared to gram-positive (Muzzarelli et al., 2000). In a study nanoparticles of chitosan-alginate were shown to activate cytokines production by boosting T-cell proliferation (Friedman, et al., 2013).

1.8.5 Chitosan's wound healing activity

Chitosan and its derivatives are highly biocompatible, biodegradable, and show antimicrobial activity with low immunogenicity, which makes them excellent materials for wound healing. Three-dimensional tissue growth matrix enhance granulation and the organization of the repaired tissues (Rhoades, and Roller, 2000). Chitosan is found to promote the activity of fibroblasts, macrophages and leukocytes and stimulate cell proliferation (Ueno et al., 2001; Jayasree et al., 1995).

Lysozymes gradually depolymerize and hydrolyze chitosan to acetyl-D-glucosamine and while preventing scar formation, collagen deposition and proliferation of fibroblasts are encouraged. Being a wound dressing matrix, chitosan displays admirable advantages such as non-toxicity, antimicrobial properties, inertness, biocompatibility, and affection to proteins (Dickinson and Gerecht, 2016). Several porous and adhesive electrospun nanofiber wound-healing material based on chitosan with high tensile strength, and oxygen transmission rate have been synthesized and tested in clinical uses such as HemCon®, ChitoFlex®, and ChitoGauze® which (Cheung et al., 2015).

1.9 Poly (3-Hydroxybutyrate-co-3-hydroxy valerate), PHBV

The polyhydroxyalkanoate (PHA)'s: are a class of biopolymers composed of hydroxyalkanoates obtained by bacterial fermentation. These biopolymers are formed in the cytoplasm in a diversity of both Gram-+ and Gram- bacteria as inclusion bodies under the poor nutritional conditions. Since they are biodegradable and synthesized from renewable carbon sources, PHAs have significant advantages when compared to petroleum-based plastics and

offer them as best alternative to conventional, non- degradable plastics. The most common and widely considered PHAs are poly3-hydroxybutyrate (PHB) and poly3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV). The chemical structure below is in Figure 1.11, PHB is the most prevalent monomer found on many chains.

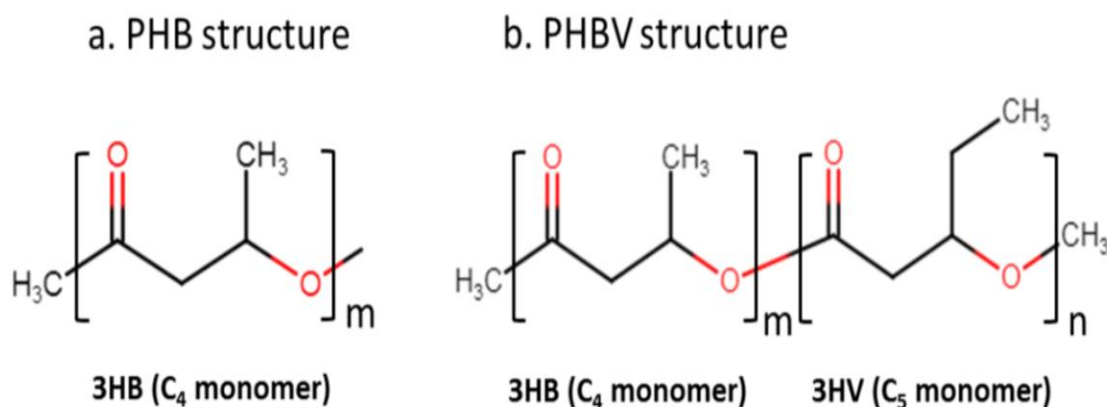


Figure 1.11: Molecular structure of PHB and PHBV (http://2018.igem.org/Team:Edinburgh_OG/PHBV_Production)

1.9.1 Biological and physical proprieties of PHBV

PHBV is 100% biodegradable, resistant to reasonable amounts of organic solvents, and oils and UV radiation proof. The physical properties of PHBV are listed in Table 1.4. However, PHBV is a rather rigid and brittle polymer having a low melting temperature, highly viscous and chemically inactive in solution. PHBV has better mechanical properties when compared to PHB, has excellent oxygen barrier, water sorption diffusion, wettability features (Ariagna et al.,2018).

Table 1.4: Physical properties of PHBV (L. Ariagna et al., 2018).

Elongation at break %	1.4
Elasticity (GPa)	2.38
Traction resistance (MPa)	25.9
Density(g/cm ³)	1.25
Fusion temperature °C	153
Glass transition temperature °C	-1

1.9.2 Biomedical applications of PHBV

Nowadays PHBV is one of candidates being used in various biomedical. It is a promising material for biomedical applications since it is not toxic and highly biocompatible with various cell types and water absorption capacity, it is precisely used in drug delivery systems. PHBV has many medical applications in tissue engineering and pharmacokinetics, and became one of the promising biopolymers (Vilos et al., 2013). Besides, not only used in encapsulating material for local drug delivery but, also benefited for the delivery of antibiotics in chronic inflammatory diseases (Qiong et al.,2009). PHBV because of its high strength, low immune response, and high mineralization capacity when compared to other biopolymers, considerably used in tissue engineering and has many applications in bone tissue and cartilage.

1.9.3 Anti-inflammatory and anti-bacterial activities of PHBV

Although PHBV has excellent properties but, it lacks antimicrobial activity and this diminishes its potential use. To deal with this problem, some additives with known antimicrobial activity such as essential oils having microbicidal activity like oregano, *Origanum vulgare* are incorporated or blended with silver nanoparticles or grafted with chitosan or chito-oligosaccharides. These nanocomposites had anti-bacterial activity to *S. aureus*, *P. aeruginosa*, and *Klebsiella P.neumonia*, and showed significant anti-viral and microbial activity to *L. monocytogenes*, *S. enterica* and *murine norovirus* (Ariagna et al.,2018).

1.9.4 Wound healing activity of PHBV

PHBV nanofibrous scaffolds obtained by electrospinning can provide a skeletal support for the wounds and help to promote the cellular events during the healing process. PHBV nanofibers have been shown biocompatible with skin tissue, and have compatible porosity and mechanical strength when compared to native human skin. When human lymphocytes were cultured on PHBV nanofibers, no inflammatory response is observed. PHBV scaffolds alone or coupled with other composites support adhesion of keratinocytes, and favor gene expression and proliferation (Sundaramurthi, 2013). Nanofibrous PHBV scaffolds modified with collagen loaded with and without unrestricted somatic stem cells (USSCs) were grafted into the skin of a rat model cells after wounds. The injuries were then examined after two weeks, extracellular matrix (ECM) recovered more quickly with the dermal strain (DS) cells attached to PHBV coupled with collagen (Keshel et al., 2014). The data also demonstrate the mechanical properties of the PHBV scaffolds which can favor the extent and regeneration rate of skin tissue.

2. Hypericum perforatum L

Belonging to *Hypericaceae* family, *Hypericum perforatum* which is also known as “St. John's wort”, history goes back to the ancient Greeks times. It has become very popular in the depression and anxiety treatment, also very effective in healing burns and cuts. *Hypericum perforatum* (Figure 1.12) is a very abundant plant that can be found in all temperate areas of the world and is the subject of much interest due to the various groups of compounds with medicinal properties (Nahrstedt and Butterwick 1997, Jarzebski et al., 2020). There are several technics being used for *Hypericum perforatum* extraction, such as soxhlet extraction, ultrasonic, solvent extraction method, and others. Compounds which are extracted are classified into several categories, the most commonly known are phloroglucinols, naphthodianthrones and flavonoids (such as phenylpropanoids, flavonoid glycosides, and flavones), pseudohypericin, hyperoside, isoquercitrin, quercitrin, miquelianin as well as essential oils (DerMarderosian and Beutler 2002; Klemow et al., 2011). Among them, hyperforin and hypericin (Figure 1.13) are the most prominent active products found in hypericum. Also, we must realize that about 20% of the compounds extracted in this plant have shown biological activities (Klemow et al., 2011).



Figure 1.12: *Hypericum perforatum L* (Klemow et al., 2011).

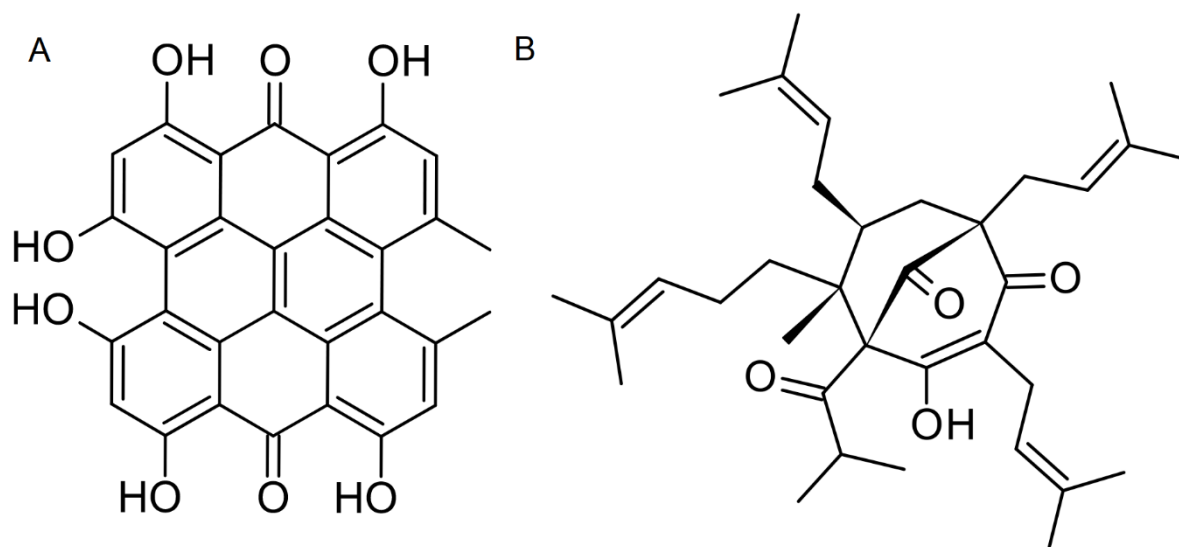


Figure 1.13: Chemical representation of hypericin(a), hyperforin(b)

2.1 Medical activities of *Hypericum perforatum* extracts

Extracts of *Hypericum perforatum* are potently used in the treatment of depression, nervousness, insomnia, and convalescence (Linde et al., 2009); Besides its uses, very efficient in curing skin diseases, slight injuries, lesions of mucosa, and gastrointestinal problems. Experiments have shown that it possesses antioxidant, anticancer, antiviral activity, antibacterial, antifungal and anti-inflammatory activities (Kenneth et al., 2011).

H. perforatum also protects the human body against free radicals and oxidative stress, which generates many diseases like Parkinson's disease, Alzheimer's, disease, cancer, diabetes and heart disease (Hemati et al., 2010). As an antioxidant, its effectiveness has been observed in rats, by modifying the levels of cerebral malondialdehyde, glutathione peroxidase, and glutathione (El-Sherbiny et al., 2003). Hyperforin and hypericin which are extracted from *H. perforatum* are responsible for inhibiting the proliferation of tumor cells and inhibit tumor growth (Belwal et al., 2018).

2.2 Anti-microbial and anti-inflammatory activity of *Hypericum perforatum*

Alcohol extracts such as butanol extract from *H. perforatum* have been shown to have antibacterial activities against *Helicobacter pylori* (Reichling et al., 2001), ethanol extracts inhibit the growth of *Penicillium canescens*, *Streptococcus mutans*, *Enterococcus faecalis* and *Streptococcus sobrinus*, (Maskovic et al., 2011). Methanol extracts also inhibit the in vitro growth of *Escherichia coli*, *S. aureus* S. mutants, *Proteus vulgaris*, *Streptococcus sanguis* and *Streptococcus-oxford* (Barbagallo and Chisari, 1987; Belwal et al., 2018). The apolar extracts

have higher anti-inflammatory activity over ethyl acetic or hydro-alcoholic extracts. Also, the two main agents from *H perforatum* extract which are quercetin and II8-biapigenin, have been detected with anti-inflammatory activity. It was shown that the extracts of *H perforatum* inhibit the expression of cyclooxygenase, interleukin 6, inducible nitric oxide synthase (iNOS) genes (Zdunic et al.,2009).

2.3 *Hypericum perforatum* extracts on wound healing

Cuts, abrasions, and other wound have been treated with extracts of *H. perforatum* from ancient times. It is well known for reducing inflammation and this activity appears to be associated, with its antibacterial activity.

H. perforatum extract usage for wound-healing has been increased in recent years. Wound closure experiments in chicken showed that embryonic fibroblasts exposed to the extract of *H perforatum* exhibited increased production of collagen, causing activation of fibroblast cells for the closure of the wound (Ozturk et al., 2007). Leaves of *H. perforatum* have also been studied on a rat model its ability to heal wounds was reported (Mukherjee et al., 2000). Besides, an increase in the re-epithelialization of the tissues in rats that had been burned was reported with a cream containing *hypericin* and the *phenytoin* cream (Sayar et al., 2014). These results clearly show that *H. perforatum* accelerates wound healing.

3. Bionanocomposites

We can define bio nanocomposites as nanocomposites made up of a biopolymer of biological origin and an inorganic solid. Among the polymers of biological origin constituting the preparation of nanocomposites we can cite collagen, chitosan, a natural polysaccharide, PHBV, and many others. Antimicrobial agents, antioxidants and cell growth factors, vitamin C or other healing materials are added to accelerate the healing features of the bionanocomposites (Figure 1.14) (Alba et al., 2019).

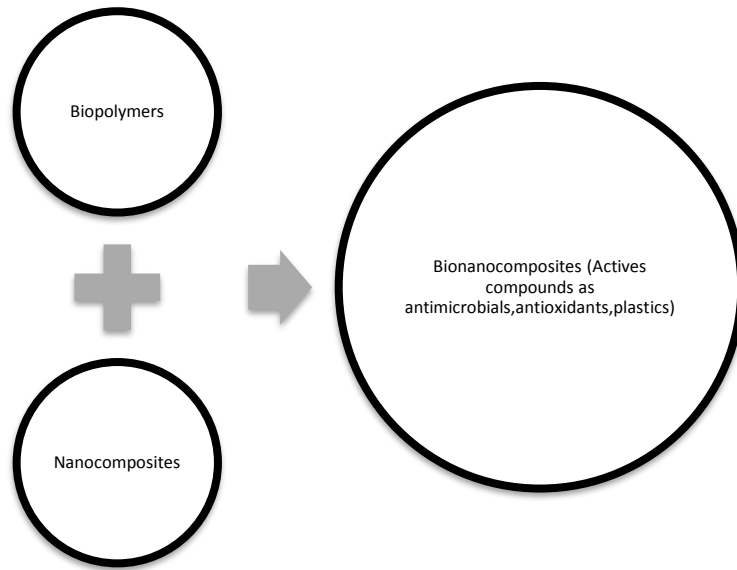


Figure 1.14: Composition of bio nanocomposites

3.1 Electrostatic spinning (electrospinning)

Electrospinning is a valuable and useful method for obtaining continued nanofibers by electric force. A basic electrospinning setup is seen in Figure 1.15 (Alharbi, et al., 2016). Although their diameter is very small, the ratio of surface area to volume of these nanofibers have a remarkable large and they showed to be have porous in nature. Therefore, it is possible to obtain many scaffolds capable of carrying bioactive substances (Wendorff, et al., 2016). All these characteristics display various applications such as biosensor and drug delivery systems, high performance intelligent textiles, tissue scaffolds. Parameters of electrospinning may be changed to obtain wound mats with distinctive characteristics (Li and Xia, 2004). This technique is mostly used these days due to the easy control during the procedure and does not require many financial means to carry it out (K. Aruchamy et al., 2018).

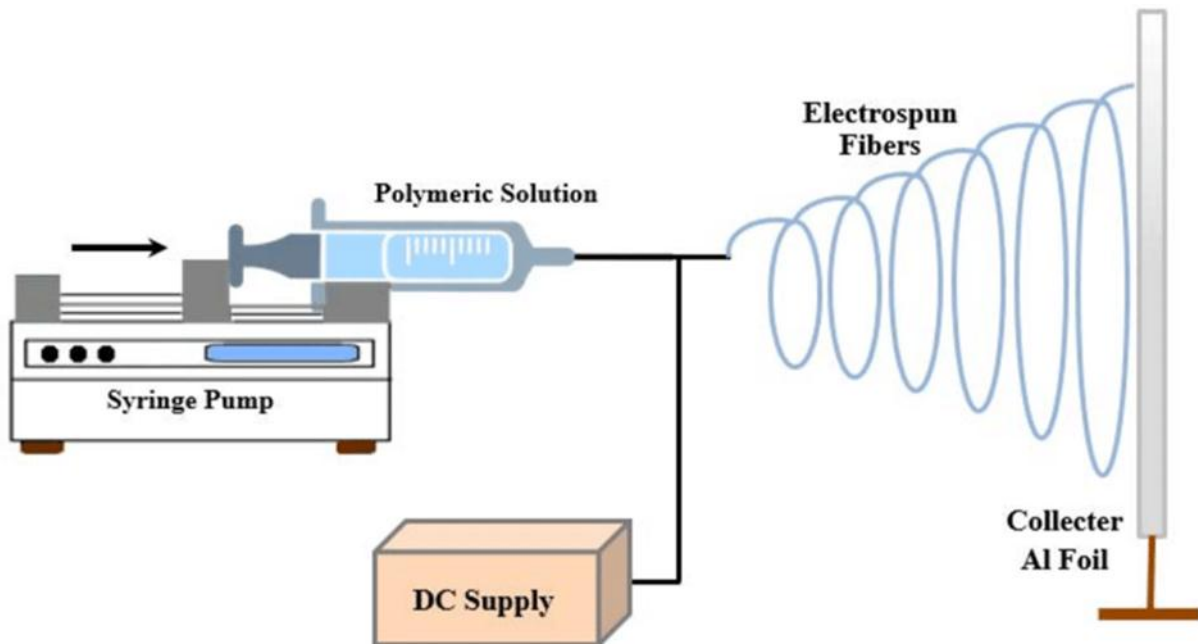


Figure 1.15: Electrospinning technique (Alharbi, et al., 2016)

3.2 Bio-nanofibers as nanocomposites

Nanostructured fibers (nanofibers), synthetic from natural and/or synthetic polymers, metals, and metal oxides, inorganic and organic carbon-based hybrid nanofibers are manufactured using several methods, the most common is electrospinning (Barhoum et al., 2019). Due to its different characteristics, electrospun nanofibers are used in several applications such as in the medical sector, including the manufacture of dressings, enzyme immobilization, and tissue-based engineering and wound healing (Sonseca et al., 2020). Nanofibers are also used in batteries as constituents of electrode and membrane materials, fuel cells, and solar cells. They also serve as membranes for ultra-high air filtration, wastewater treatment, water purification, and low-pressure blood purification which were shown in Figure 1.16 (Barhoum et al., 2019). Also, polymeric nanofibers from electrospinning method have an interconnected three-dimensional network and have a high porosity (Coelho et al., 2018)

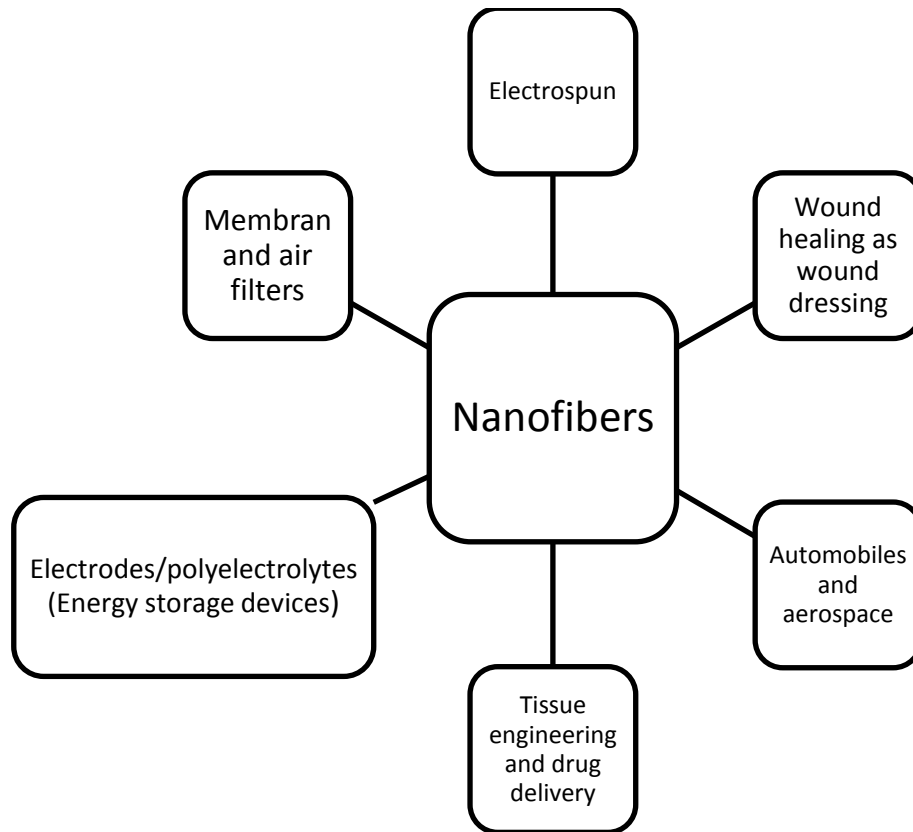


Figure 1.16: Electrospun nanofiber applications (Aruchamy et al., 2018)

3.3 Approach of using bio-nanofibers on wound healing treatments

For several years, nanofiber polymers have been diversely used in the production of pharmaceuticals and in the treatment of wounds. To have a controlled drug release in wounds, nanofiber polymers produced by electrospinning scaffolds composed of fibroblast growth factor (FGF) and epidermal growth factor (EGF), as plant extracts and factors inducing angiogenesis have been utilized to improve healing process. These nanofibers have accelerated the *in vivo* healing studies. Some nanofiber scaffolds modified with gelatin enhanced the dermal fibroblasts proliferation and the infiltration in the designed scaffolds and induced the excretion of ECM proteins, providing information for the potential dermal tissue repair (Coelho et al., 2018). In Figure 1.17, the flow chart of fabrication of nanofiber was given.

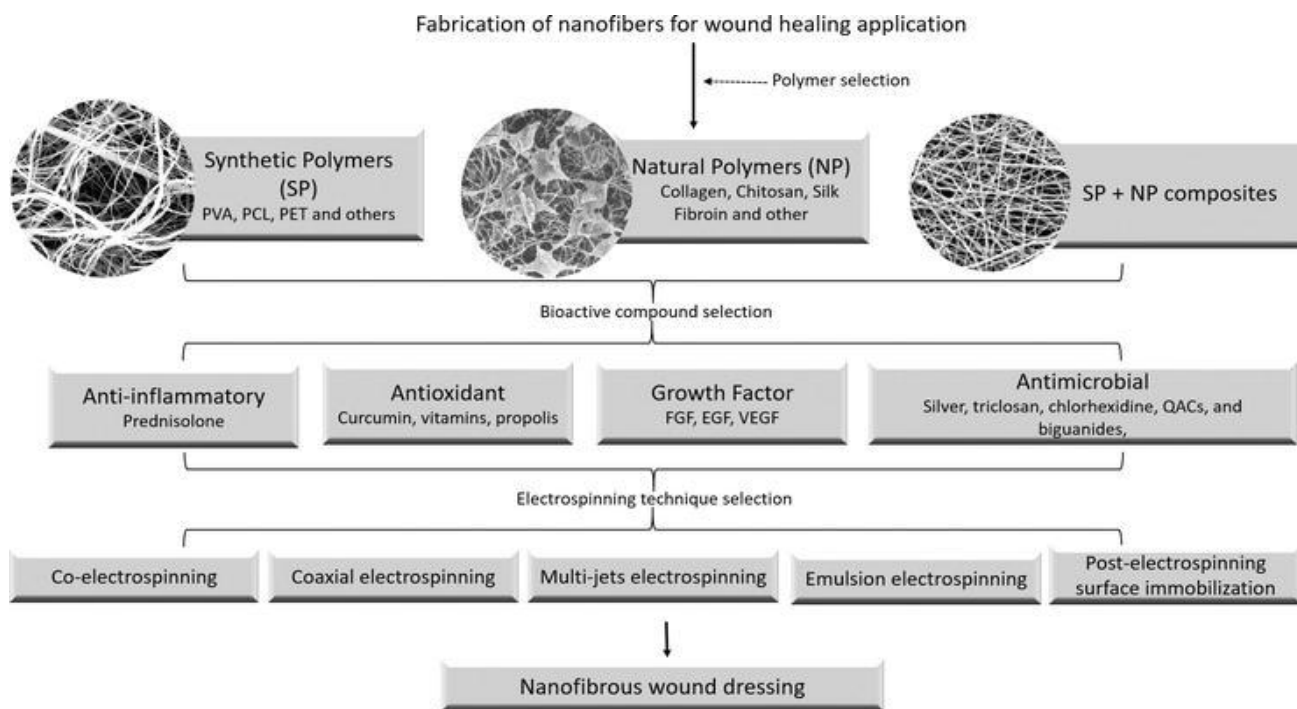


Figure 1.17: Fabrication of nanofibers (Coelho et al., 2018).

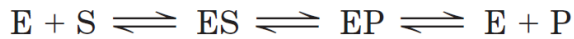
4. Collagen, chitosan, and PHBV as bio-nanofibers

Electrospun nanofiber polymers with a composition of bioactive compounds for the skin tissue engineering, facilitates the healing of numerous skin disorders characterized by the appearance of wounds. Nanofibers must have several characteristics such as, low cytotoxicity, good porosity, oxygen permeability, degradability. They must prevent against bacterial proliferation, favor accelerated re-epithelization at the site of the injured wound and promote the growth of keratinocytes and fibroblasts which are important in the wound healing process (Coelho et al., 2018). Biocompatibility of electrospun collagen/chitosan nanofibers and their cellular behavior on nanofibrous scaffolds were examined. Morphologies were examined by FTIR, SEM, and the X-ray diffraction techniques. Observations reveal that collagen-chitosan nanofiber is very promising in biomedical applications especially in tissue engineering studies (Chen et al., 2010). Chitosan nanofiber combined with green tea extracts have been prepared by electrospinning method to improve antimicrobial activity and accelerate wound healing (Sadri et al., 2015). A chitosan-crosslinked biodegradable electrospun PHBV nanofibrous scaffold was designed as nerve conduit by Biazar and Keshel. The analyses and cell culture assays displayed a neural graft with good compliance and resiliency with movement (Biazar and Keshel, 2014).

As a result we can conclude that nanofibers can be an ideal for wound dressing.

5. Enzymes

Enzymes are biological catalysts except a small group of ribozymes generally protein in nature. Enzymes are extraordinary catalyzers, their catalyzing power is much higher than inorganic catalysts and very specific for their substrates and extremely increases biological reactions. They can function at mild conditions of temperature and pH,



Enzyme; E, substrate; S, product; P, ES and EP are transient complexes. Many enzymes follow Michealis Menten kinetics which is expressed by the Michaelis-Menten equation.

$$V_0 = \frac{V_{\max}[S]}{K_m + [S]}$$

The Michaelis-Menten equation can be mathematically transformed into Lineweaver-Burk plot (Figure 1.18) that is practical K_m and V_{\max} determination.

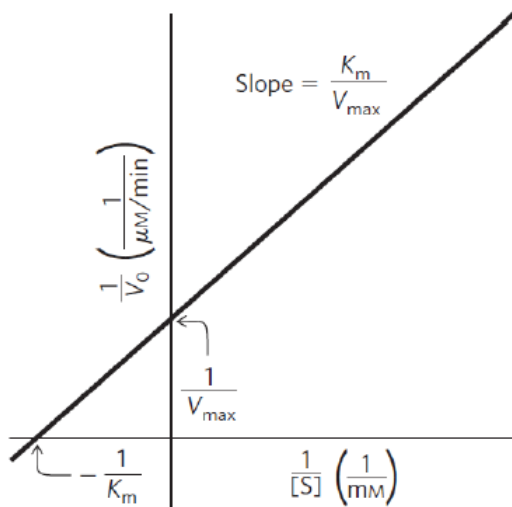


Figure 1.18: Lineweaver-Burk plot (Lehninger et al., 2005)

5.1 Enzyme inhibition

Inhibitors are molecules which can slow or sometimes stop the enzymatic reactions. Inhibitors can be classified as irreversible and reversible. Irreversible inhibitors bind very firmly to the active center of an enzyme and destroy the active center that is essential for enzyme activity. But, a reversible inhibitor does not bind firmly, can easily leave the enzyme. Reversible inhibitors can be typed as; competitive, uncompetitive and mixed type

Competitive inhibition of enzymes

A competitive inhibitor competes with the substrate for binding to active center . Inhibitor (I) binds to the active site and inhibit the substrate binding to the active site. V_{max} does not change but K_m increases (Lehninger., 2005) (Figure 1.19).

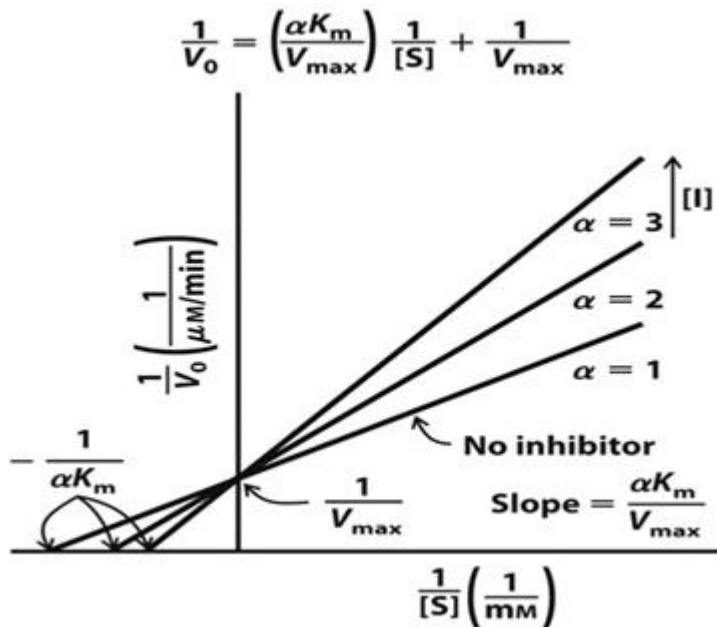


Figure 1.19: Competitive inhibition of enzymes (Lehninger et al., 2005)

Uncompetitive inhibition of enzymes

An uncompetitive inhibitor does not compete with the substrate, binds to the active site other than substrate binds. binds and also to ES complex. Both K_m and V_{max} decreases (Figure 1.20).

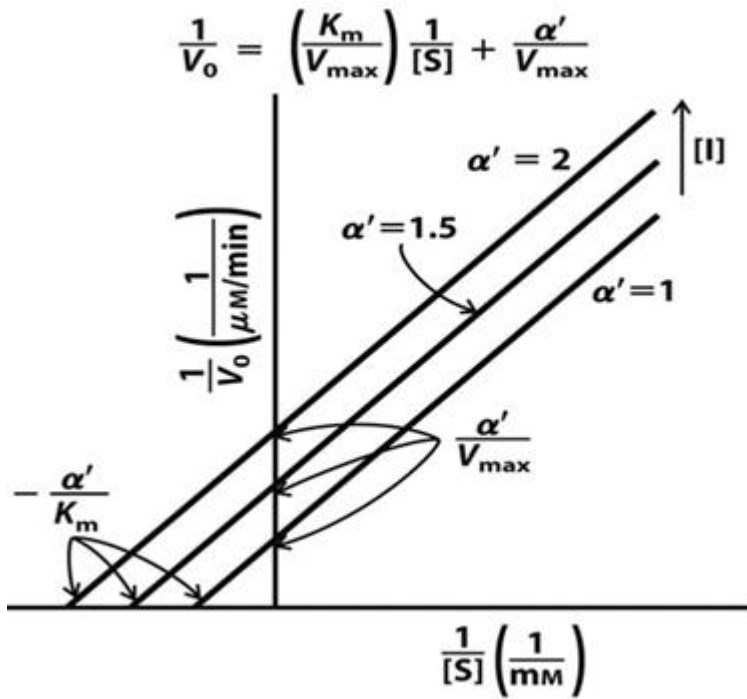


Figure 1.20: Uncompetitive inhibition of enzymes (Lehninger et al., 2005)

Mixed inhibition of enzymes

The inhibitor shows the properties of both competitive and uncompetitive inhibition. It may bind both to E or ES. K_m increases but V_{\max} decreases (Figure 1.21), (Lehninger., 2005).

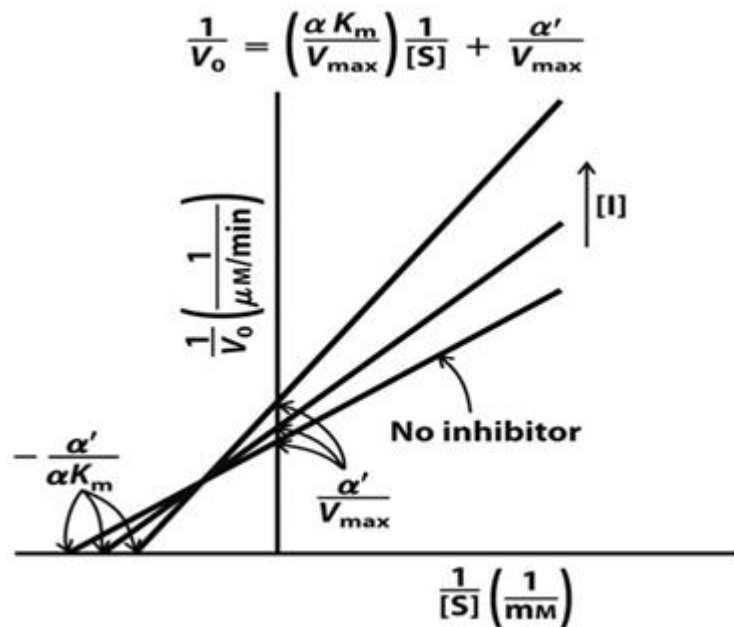


Figure 1.21: Mixed type inhibition (Lehninger et al., 2005)

6. Collagenases

Proteases can cleave the peptide bonds into small peptides and amino acids. Collagenases are site specific proteases whose main roles are to degrade collagen. Collagens are very resistant to the proteases because of their rigid triple helix structure but can be easily cleaved by collagenase enzyme (Figure 1.22) (Pal and Suresh 2016). Collagenases which have different substrate specificities are mostly found in various species such as animals, plants and microorganisms. Collagenase from eukaryotic sources are site specific but prokaryotic bacterial collagenases are not (Adhikari et al. 2012; Baehaki et al. 2012). Collagenase is commonly isolated from *Clostridium histolyticum* and bacterial collagenase is unique because it degrades both insoluble collagens and denatured collagens soluble in water and is capable of making several cleavages in regions with triple helix (Mookhtiar and Van Wart 1992). Collagenases from microbial sources have vast biomedical applications such as restorative applications including wounds treatments, besides their predominant medical applications, collagenases are also used in food industries such as fish and meat processing, beer clarification and stabilization, brewing and in scientific research (Bhagwat and Dandge 2018; Pal and Suresh 2016). Collagenase expression is very critical in wound healing, high or long expression can result in enormous ECM degradation with defective curing (McCarty and Percival, 2013). Prolonged inflammation is the common feature of all chronic wounds. Inflammatory cells continuously influx into the wound site where they undergo apoptosis. There is a continuous death of inflammatory cells and their secondary metabolites accumulate. The level of the released proteolytic enzymes which are able to degrade the dermal tissue, essential growth factors and receptors increase. But it was reported that if the neutrophil-derived collagenases are plenty in the wound area, then wound repair would be slow. It was shown that wound repair was stimulated after the partial inhibition of the collagenases (Francesko et al., 2013).

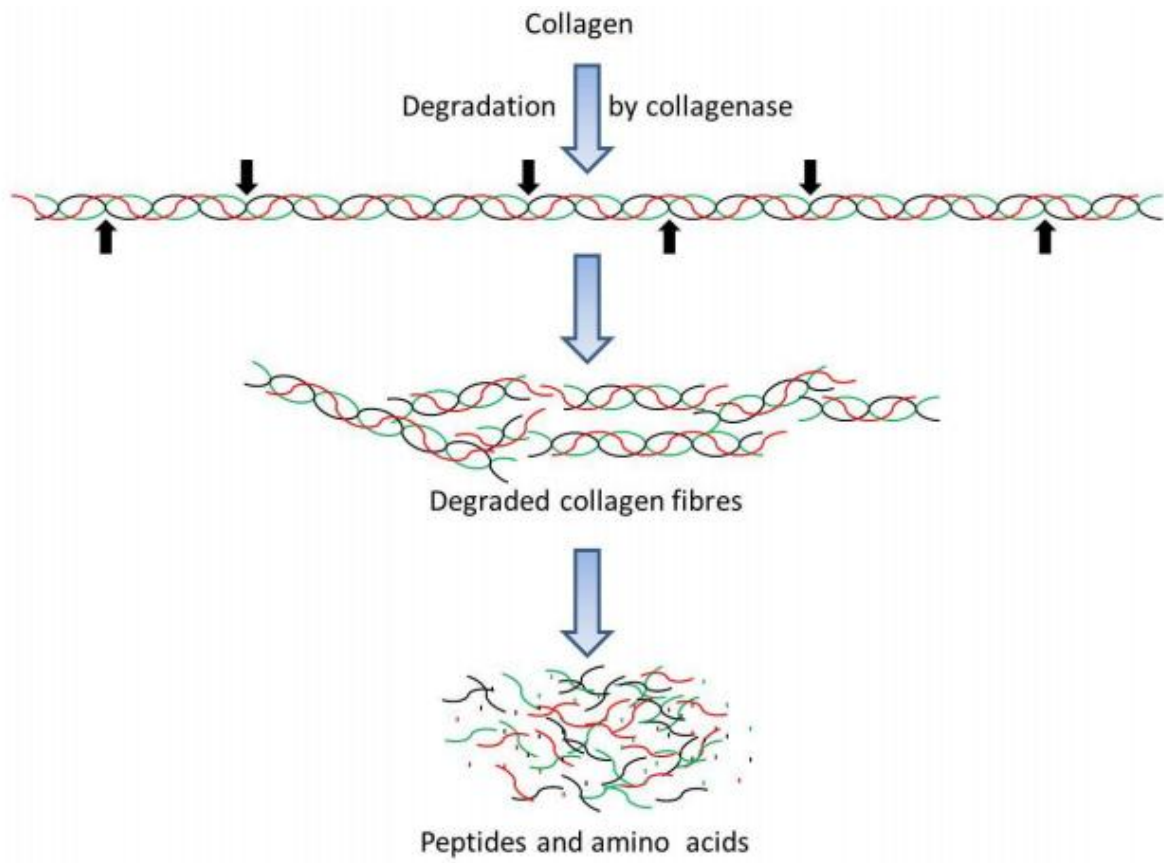


Figure 1.22: Degradation of collagen ((Pal and Suresh 2016).

II. MATERIAL AND METHODS

2.1. Methodology

Brief explanation as a flow chart has been summarized in Figure 2.1.

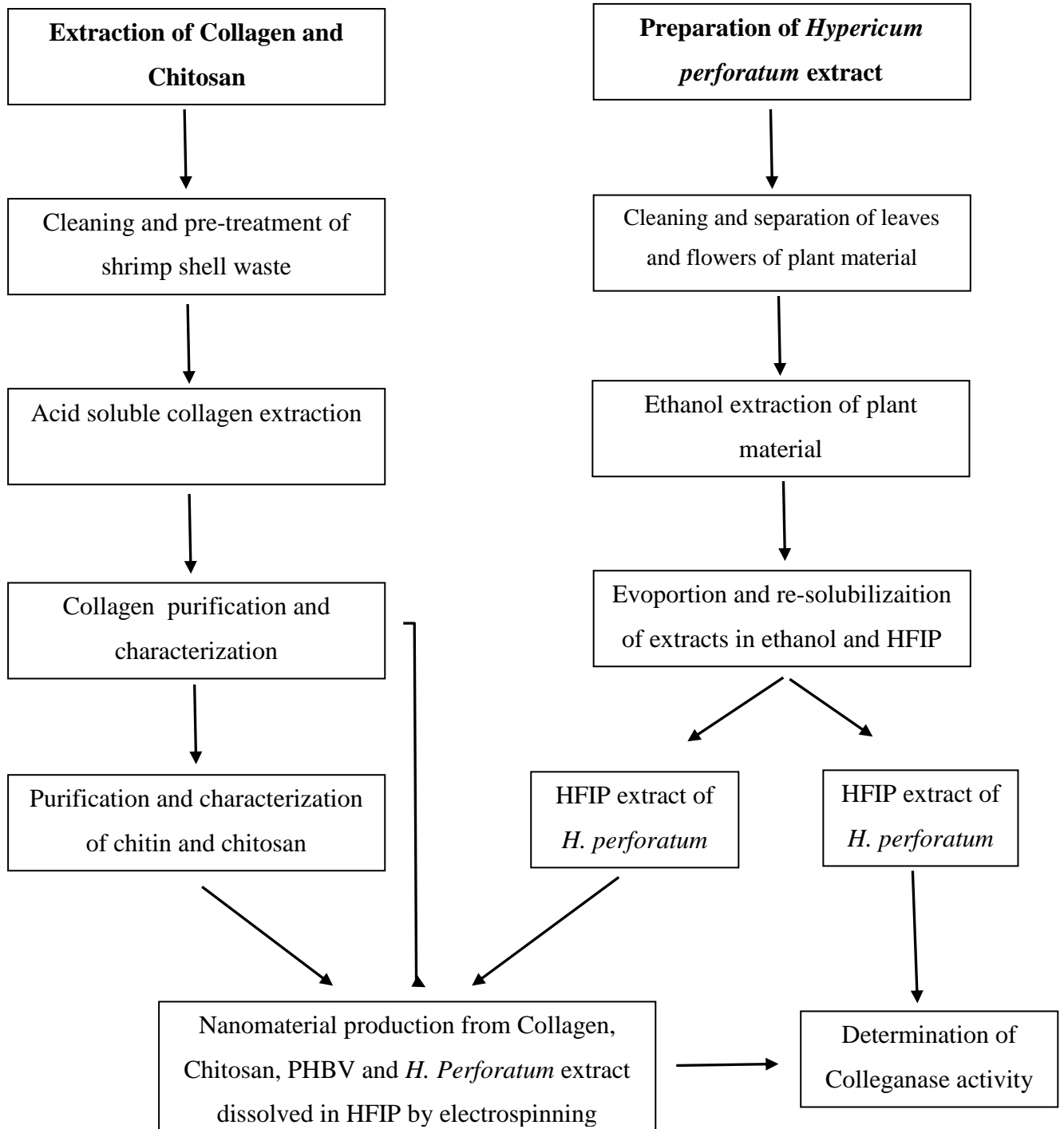


Figure 2.1: Schematic representation of research methodology

2.2 Apparatus

FTIR:	Perkin Elmer ATR–FTIR. (U.S.A)
4 °C Fridge:	Arçelik (Türkiye)
Orbital Shaker:	IKA HB 10 and IKA RV 10 digital (US)
-20 °C refrigerator:	Arçelik (Türkiye)
-86 °C Freezer:	Thermo Electron Corp., Forma ULT (U.S)
Centrifuge:	Sigma Laboratory Centrifuges, 3k30 (Germany)
Distilled water appliance:	Millipore, (Germany)
Electrophoresis power supply:	Apelex, PS9009 TX (France)
Electrospinning apparatus:	Inovenso, NE3 (Turkey)
Magnetic stirrer:	Chiltren, HS 31 (UK)
pH meter:	Toledo Mettler, S20 (Switzerland)
Pipettes:	Gilson (U.S)
SEM:	Philips XL30 ESEM-FEG/EDAX (Netherlands)
Spectrophotometer:	Thermo Scientific, UV-VIS (U.S)
Vortex machine:	Fisons, (UK)
Water bath:	Nüve (Türkiye)
Weighing/ Precision balance:	Sartorius Analytic, A200S ± 0,0001 (Germany)
Electrophoresis system:	Bio-Rad (U.S)

2.3 Chemicals

1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), Poly-3-hydroxybutyrate-3-co-hydroxyvalerate (PHBV), Bradford reagent, bovine serum albumin (BSA), wide range SigmaMarker and bromophenol blue were obtained from Sigma, sodium chloride, acetic acid, sodium hydroxide glycerol, disodium ethylenediaminetetraacetate (Na₂EDTA), acetone, copper sulfate, sodium carbonate, urea, sodium dodecyl sulfate (SDS), acrylamide, N, N'-methylenebisacrylamide (bis-acrylamide), ammonium persulfate (APS), N, N, N', N'-tetramethylethylenediamine (TEMED), ethanol, sodium thiosulfate, formaldehyde, methanol were purchased from Merck.

2.4 Collagen and Chitosan extraction from Shrimp Shell Waste

Collagen and chitosan extraction from the shrimp shell material has carried out according to Odabas, 2020 with some changes.

2.4.1 Pre-treatment of samples

Frozen shrimp shell waste was obtained from Sagun Group. Tekirdağ fish processing factory. In order to prevent heat degeneration of biomaterials all experiments were carried out in the temperature controlled room on an orbital shaker at 4 ° C.

Shrimp wastes are washed several times with excess of cold water and the visible impurities are removed by hand. Obtained shrimp shells was allocated into 500 grams and kept at -80 ° C until experiments was carried out.

2.4.2 Removal of non-collagenous proteins, minerals and fatty substances

Pre-treated shrimp shell waste treated with the consecutive washes with alkaline solution in order to remove non-collagenous proteins. For this purpose, 250 g of shrimp shell waste washed with 1000 mL of 0.1 M NaOH solution on an orbital shaker with a rotating speed of 150 rpm at 4 ° C for 24 hours. After the filtration of the solution, process was repeated with 1000 mL of 0.1 M NaOH solution.

Obtained solid material washed with pure water until neutrality is reached and treated with 500 mL of 0,5 M Na₂EDTA on an orbital shaker with a rotating speed of 150 rpm at 4 ° C for 24 hours. This process is repeated twice for the complete removal of remaining non-collagenous materials and minerals such as Ca²⁺ and Mg²⁺.

Oils and fatty substances in the pre-treated shrimp shell waste material has been removed by the addition of 250 mL 10% n-butanol on an orbital shaker with a rotating speed of 150 rpm at 4 ° C for 24 hours. After the process completed, waste materials washed and dried at room temperature for 3 hours and weighed.

2.4.3 Collagen extraction from shrimp shell waste

In order to extract acid soluble collagen from the pre-treated and dried shrimp shell waste material 180 mL 0,5 M acetic acid on an orbital shaker with a rotating speed of 150 rpm at 4 °

C for 24 hours. Obtained raw collagen extract was centrifuged at 12000 g for 60 minutes for the removal of non-soluble materials and supernatant collected for the collagen precipitation. On the other hand, precipitate from the centrifuge process was dried at 80 °C for 2 hours, ground, weighed and used for chitosan production.

In order to precipitate collagen, solid sodium chloride was slowly added to the supernatant at 4 °C until a 9 M concentration is reached and the mixture incubated for 24 hours at 4 °C with constant stirring. Then mixture centrifuged at 12000 g for 60 minutes and after supernatant was removed precipitated collagen dissolved in minimal amount of 0,5 M acetic acid. Salts removed from the collagen with dialysis against 0,5 M acetic acid (24 hours), followed by 0,1 M acetic acid (24 hours) and distilled water (24 hours). At the end of the dialysis dialysate lyophilized in a glass petri dish and stored at -80 °C until further use.

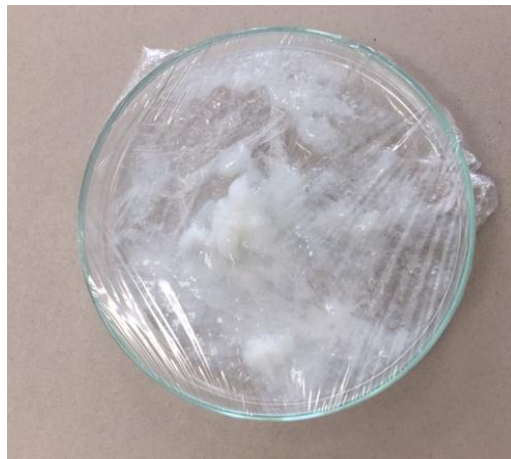


Figure 2.2: Lyophilized collagen from shrimp shell waste material

Bradford method was used to determine the protein concentration of the collagen sample (1976). Briefly 0.2 g of sample material was dissolved in 10 mL 0.05 M acetic acid and 2 μ L of this solution was added to 798 μ L of distilled water. After the addition of 200 μ L of Bradford reagent sample was incubated for 20 minutes and developed color was determined spectrometrically at 595 nm. Using a standard calibration graph for known concentration of BSA protein concentration was determined.

According to Rath et al (2013) method shrimp shell waste collagen has been characterized by SDS-PAGE with 4% stacking (3 mL of 29.2% acrylamide/0.8% bisacrylamide; 2.5 mL of 0.5 M Tris-HCl pH 6.8; 200 μ L of 10% SDS; 15 mL of distilled water; 70 μ L of ammonium

peroxysulfate; 20 µL of TEMED) and 8% separation gel (26 mL of 29.2% acrylamide/0.8% bisacrylamide; 16.25 mL of 1.5 M Tris-HCl pH 8.8; 650 µL of 10% SDS; 16.25 mL of distilled water; 350 µL of ammonium peroxysulfate; 70 µL of TEMED) with BIO-RAD Mini electrophoresis system according to the manufacturer's instructions.

Collagen sample was dissolved in minimal amount of 0,5 M acetic acid and equal amount of PAGE buffer (1 mL of 1M Tris-HCl pH8.8; 1.6 mL of glycerol; 200 µL of 10% SDS, 400 µL of 25% DTT; 8 mL of distilled water and trace amounts of bromphenolblue) was added and boiled for 4 minutes. Sample or standard protein marker was added to the wells (35 µg protein per well) and electrophoresis was carried out in the electrophoresis buffer (0.3% Tris-Base, 1.5% glycine, 0,01% SDS) at 30 mA per gel. When the electrophoresis completed, gel was removed from the cassettes, washed, fixed (10% acetic acid, 50% methanol) and stained with Coomassie stain solution.

2.4.4 Chitin extraction and chitosan synthesis from shrimp shell material

De-collagenized, dried and ground shrimp shell waste material decolorized with 100 mL pH10 5% H₂O₂ (w/v) solution at room temperature, overnight. Resulting material washed until neutralization and further demineralized with 1N HCl at room temperature for 24 hours. Chitin was obtained by filtration and incubation of the solid material at 160 °C for 4 hours.

Chitosan was produced alkaline deacetylation of chitin. 2 g finely ground chitin was added to a 100 mL 50% NaOH and refluxed overnight at 80 °C with constant stirring. Resulting chitosan first washed until neutralization with distilled water, then 80% ethanol and dried.

Chitosan characterized by its deacetylation degree using acid-base titration method. 0.1 g chitosan dissolved in 30 mL 0.1 M HCl and titrated with 0.1M NaOH in the presence of methyl orange. Deacetylation degree calculated using the formula;

$$DD = \left(\frac{(C1 \times V1 - C2 \times V2)}{m} \times 0.0094 \right) \times 0.016$$

Where C1 and V1 are HCl concentration and volume, C2 and V2 are NaOH concentration and volume, m is the weight of the chitosan.

2.5 Preparation of *Hypericum perforatum* (sarı kantaron) extract

Plant material of *Hypericum Perforatum* was harvested from Mersin region during flowering season during June 2019 and plant sample was identified at the Marmara University Biology Department. Upper parts of the plant material were dried in room temperature, then flowers were manually separated and ground into small pieces. 100 g of homogenized plant material was macerated with 1400 mL 98% ethanol in 2L glass beaker at 100 RPM constant mixing on an orbital shaker at room temperature for 3 days. Obtained extract was evaporated to dryness with IKA HB 10 rotary vacuum evaporator and weighed.



Figure 2.3: *Hypericum perforatum* plant material and evaporation

In order to prepare stock extract solution 5g of dried *Hypericum perforatum* extract was dissolved in 50 mL of ethanol (100 mg dry weight/1 mL extract) and this extract was used in the enzyme activity experiments.

2.6 Production of nanomaterial

The electrospinning has been done according to the method of Unnithan et al (2018).

2.6.1 Electrospinning of PHBV/Collagen/Chitosan and *Hypericum perforatum* extract

Electrospinning of the PHBV/Collagen/Chitosan and *Hypericum perforatum* extract was done using HFIP solvent. PHBV (0.4g /10 mL), collagen (0.4g /10 mL), chitosan (0.4g /10 mL) and *Hypericum perforatum* dried extract (0.4g/10 mL) solutions were prepared separately and mixed overnight. Then solutions were mixed in an 100 mL glass bottle and mixed for 2 hours

to ensure complete homogenization and resulting solution was taken with an injector fitted with a metal needle. Conditions of electrospinning were; 18 kV voltage, 15 cm of distance and 04 mL/hour pump rate. In the electrospinning machine, the jet of polymer emerges from the needle to form a cone in the plaque known as the Taylor cone due to the force of the electric field. Nanofiber mat was collected from the aluminium foil and stored at room temperature until further use.

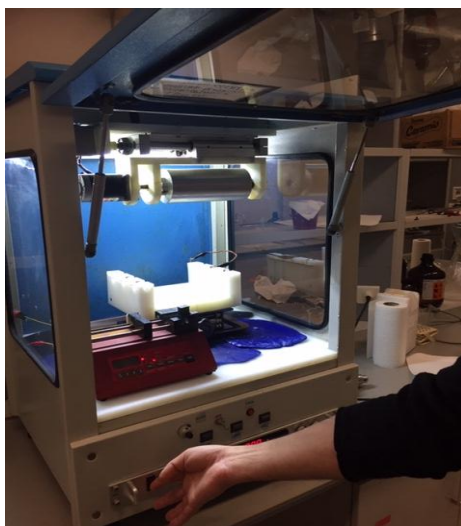


Figure 2.4: Electrospinning process

2.6.2 Characterization of composite material

An FT-IR spectrometer (Perkin Elmer Spectrum100 ATR-FTIR) was used to obtain IR spectra and the morphology of the nanomat was determined by using SEM (Philips XL30 ESEM-FEG/EDAX).

2.7 Determination of Collagenase Activity

2.7.1 Collagenase inhibitory activity of *Hypericum perforatum* extract

Collagenase inhibitory activity was determined according to the method of Ito et al., with small changes (2018). Briefly, 2.5 μL of plant extract, 22.5 μL of ethanol, 25 μL of collagenase suspension solution (26 units/mL) in 100 μM Tris HCl and 0.2 mL of 0.5 mg/mL substrate solution (phenylazobenzoyloxycarbonyl-Phe-Leu, PZ-peptide) was incubated for 30 min at 37°C. After the addition of 2.5 mL ethyl acetate and 500 μL 0.025 M citric acid, mixture was centrifugated at 2500 rpm

After the incubation, 0.5 ml of 25 mM citric acid and 2.5 mL of ethyl acetate was added and resulting solution was centrifuged at 2500 g for 10 minutes at 8 °C and the absorbance of the ethyl acetate layer was determined at 320 nm. Experiment was repeated with the addition of 2.5 µL of ethanol instead of plant extract to determine the enzyme activity without the inhibitors. Inhibitory activity determined using the formula;

$$\% \text{ Inhibition} = [(A_0 - A_s) / A_0] \times 100$$

Where A_s is the absorbance of the sample with the *Hypericum perforatum* extract and A_0 is the absorbance of the sample without *Hypericum perforatum* extract. Experiment was performed in triplicate.

2.7.2 Effect of PHBV/Collagen/Chitosan/*Hypericum perforatum* extract nanomaterial on collagenase activity

Effect of PHBV/Collagen/Chitosan/*Hypericum perforatum* extract nanomaterial on collagenase activity was determined as described as 2.7.1 by using 0.1 mg nanomat material (containing 4 mg *H. perforatum* extract) instead of 2.5 µL of plant extract. Experiment was performed in triplicate.

III. RESULTS AND DISCUSSION

3.1 Collagen and chitosan extraction from shrimp shell waste

250 g wet shrimp shell waste material was cleaned and pre-treated to remove non-collagenous proteins, minerals and fatty substances as described in section 2.4. After the process, 43 g of dried shrimp shell waste material was obtained for the extraction of collagen and chitosan. Yield was determined as 17.2% of the wet shrimp waste. In Figure 2.2 Lyophilized collagen from shrimp shell waste material was shown. Waste samples obtained from the fish processing plant contains high water from process and shrimp material therefore the yield considered optimal when comparing with the similar studies in the literature. Various collagen yields from sea chub (3.4%), eagle ray (5.3%), red stingray (5.7%), cuttlefish (2%) and paper nautilus (5.2 %) has been reported in the literature and our yield can be considered higher than this results (Boudouaia et al., 2019, Raghavankutty et al., 2017). Collagen extraction was performed by using acetic acid as described in section 2.4. and the amount of obtained collagen was

determined as 0,68 g. In the literature it has been reported that 10-12% of the shrimp shell weight is collagen. Our results confirm the literature (Hose et al., 2014).

Collagen produce films, which find vast use in wound dressings and drug delivery system. However, alternative collagen sources are needed other than mammalian sources because of the various diseases such as epidemic of mad cow disease. Fish collagens seem to be promising candidates, vast quantities of protein containing byproducts in fish industry are disposed as waste (Shahidi et al., 1995). Fishery bioprocess industry, is a great potential for utilizing most of these waste into valuable products. Marine shrimps which are commercially important are widely distributed throughout the world, mainly exploited in tropical, subtropical, and warm temperate waters.

Characterization of collagen was done by SDS-PAGE as described before (section 2.4.3) and resulting gel image was given in Figure 3.1. SDS-PAGE was performed on collagen isolated from shrimp shell and was evaluated according to the molecular weight marker. Electrophoretic pattern obtained for acid collagen displayed at least two α chains ($\alpha 1$ and $\alpha 2$) and the β chain was the major constituent in collagen, the molecular mass of $\alpha 1$ chain was around 116 kDa and that of $\alpha 2$ was very close to it. The β chain had a molecular mass of nearly 200kDa. The presence of two different subunits in the electrophoretic pattern reminds that the isolated collagen from the shrimp shell may be type 1 collagen. Comparable results for Cobia skin were also reported (Raghavankutty et al., 2017), *Thunnus alalunga*, *Pomadasys kaakan* (Noitup et al., 2005), *Pogonia cromis* (Ogawa et al., 2004). Type I collagen which is the major components of fibrous collagen is the most abundant collagen (Wu., et al., 2011).

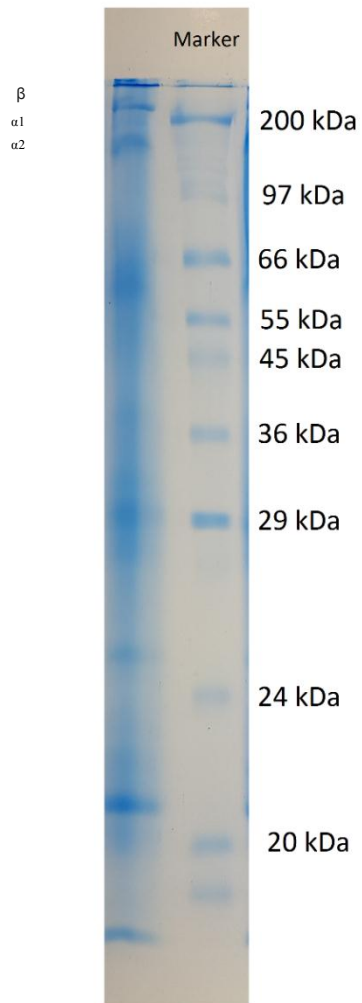


Figure 3.1: Collagen SDS-PAGE obtained from shrimp shell waste material

Protein content of the obtained collagen sample was determined Bradford method and standard calibration curve was given in Figure 3.2. Protein concentration of the sample was found as 8.37 mg/mL.

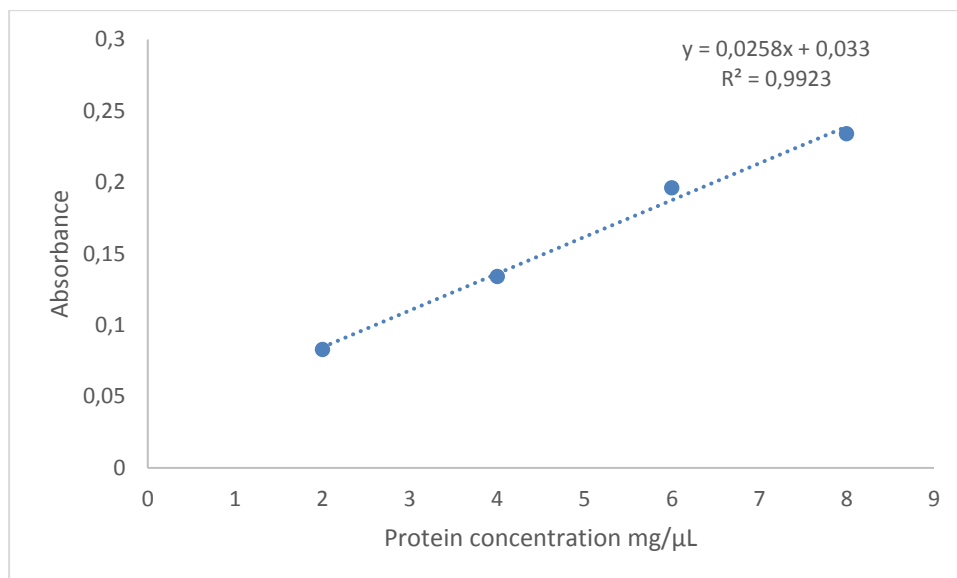


Figure 3.2: Standard calibration graph used for protein determination

3.2 Chitin extraction and chitosan synthesis from shrimp shell material

Chitin obtained from the shells of crustaceans, chiefly crabs and shrimps are the major source of chitosan. Shrimp shells are thinner so, the purification process is easier. In the purification process, shells are arranged and grouped according to their sizes. Then, they are thoroughly washed. grounded after drying. Chitin sources are very diverse so, there is no standard purification method. Proteins, pigments and minerals that may remain during the purification process must be removed otherwise these can cause severe adverse effects if they are intended for biomedical or pharmaceutical uses (Cheung et al., 2015). In our study we preferred chemical deacetylation process since it is more economic and feasible for mass production and commercial preparation (Cheung, et al., 2015). Derivatives or composites are prepared after chitosan reacting with other small molecules or polymers. Chitin extraction from de-collagenized, dried and ground shrimp shell waste material was described in section 2.4.4. Shrimp shell waste material used for chitin extraction was weighed as 34.3 g and 27.1 g of chitin was obtained after the extraction process with a yield of 79.01%. 2g chitin obtained from this process was used for the production of chitosan as described in section 2.4.4. Obtained chitosan was weighed as 0.86g (yield 43%). Deacetylation percent of the chitosan was determined as 87.83 %. There are various yields and deacetylation percentages have been reported in the literature.

Our results confirm other similar studies of Teli and Sheikh in which chitosan was extracted from shrimp shells waste and applied in antibacterial finishing (Teli and Sheikh, 2012). In another study; nanofibrous and adhesive-based chitosan have been developed as a wound dressing. studies (Cheung et al., 2015). Nowadays there are significant number of chitosan-based non-wovens wound dressings in the market. Wound healing performance is highly promising. Encouraging results were reported for the use of chitosan in clinical studies.

3.3 Electrospinning of PHBV/Collagen/Chitosan and *Hypericum perforatum* extract and characterization

High number of different polymers carrying numerous bioactive substances can be used for obtaining scaffolds by electrospinning. Changing the configuration and parameters of the electrospinning process results in different morphologies. Chitosan nanofiber mats produced by the electrospun process are porous, have a high tensile strength, high surface area with ideal wound healing features such as ideal humidity and oxygen transmission rate. They are shown to be beneficial for wound healing (Naseri, et al., 2014). In our study combination of PHBV, collagen, chitosan and *Hypericum perforatum* extract may benefit from the features of these materials and improve the healing capacity of the wound dressing.

FTIR spectra of PHBV, *H. Perforatum* extract, electrospun PHBV/Collagen/Chitosan and PHBV/Collagen/Chitosan/*Hypericum perforatum* extract material were given in Figure 3.3. According to this FTIR spectra; peak belong to NH_2 groups in chitosan and collagen peak could be seen in 3321 cm^{-1} . In addition, the peaks observed at about 2977 and 2873 cm^{-1} coincide to C-H bands of $-\text{CH}_2$ and $-\text{CH}_3$ groups in PHB. The distinctive ester carbonyl group of PHBV appeared at 1734 cm^{-1} .

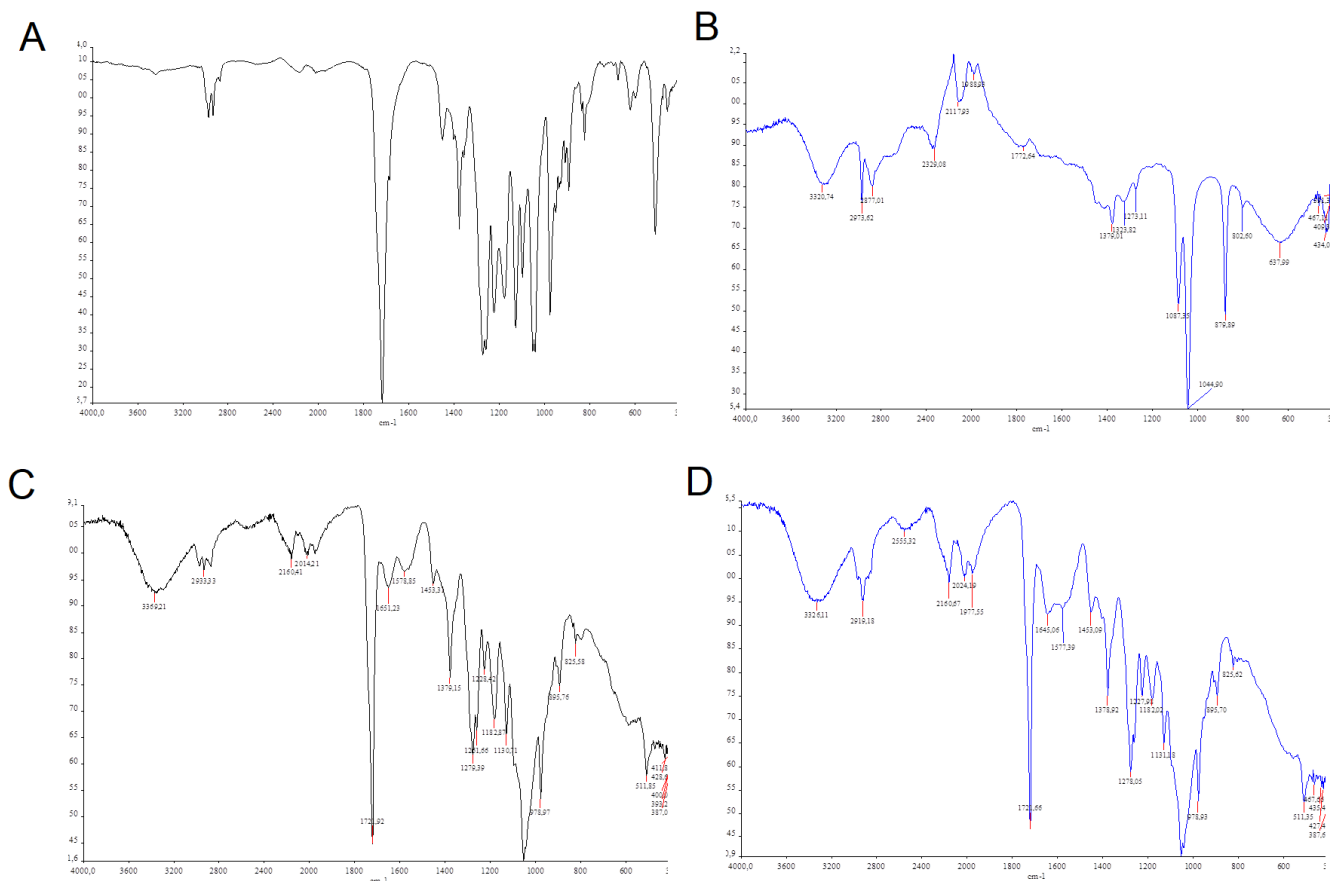


Figure 3.3: FTIR spectra of A-PHBV, B- *Hypericum perforatum* extract, C- Collagen/Chitosan/PHBV material, D- Collagen/Chitosan/PHBV/*Hypericum perforatum* extract material

SEM image of the electrospun Collagen/Chitosan/PHBV/ *Hypericum perforatum* extract material was given in Figure 3.4

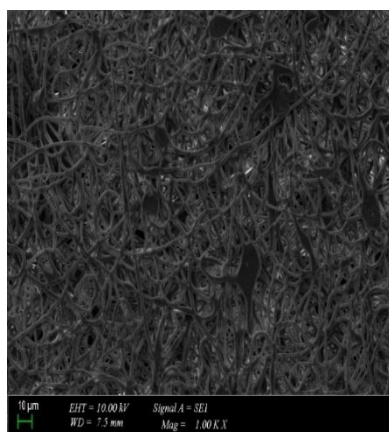


Figure 3.4: SEM image of Collagen/Chitosan/PHBV/ *Hypericum perforatum* extract material

In our study, the fiber diameter in collagen/ chitosan/ PHBV/ *H. Perforatum* extract composite material is found to be between 100-225 nm. Similar results found in the literature (Odabas 2020).

3.4 Determination of Collagenase Inhibition Activity

Since *Hypericum perforatum* extract having anti-inflammatory, antibacterial, antioxidative and anti-collagenase effects has distinctive biological and pharmacological features. Therefore, it is added to nanofibers scaffolds to improve the healing effect. The collagenase inhibitory activity of alcohol extracts of *Hypericum* species might have different mechanisms. Polyphenols have been shown to exhibit substantial collagenase inhibition. It is suggested that the hydroxyl groups of polyphenols accept or give hydroxyl groups to the enzyme amide groups and interacts with other key residues (Madhan et al., 2007). Moreover, the hydrophobic interaction between the enzyme and the benzene group of polyphenolic compounds can lead to conformational changes (Hartanto et al., 2019) and as consequence, enzyme is inhibited. Chlorogenic acid which is the richest phenolic acid in hypericum extracts showed a higher inhibitory effect (Ersoy, E., et al., 2019). Effect of *Hypericum perforatum* extract on collagenase activity has been explained in section 2.7.1. Percent inhibition of the *Hypericum perforatum* extract at 0.25 mg/ mL concentration on the collagenase was found as 89.3%, In a study *Hypericum hircinum* extract which is another species of Hypericum genus inhibited collagenase 90% at the same concentration (0.25 mg/ mL) Mandrone, M.,et al., 2015) and another study showed that the methanol extract of *H. perforatum* inhibited collagenase activity with IC₅₀ values of $61.53 \pm 0.40 \mu\text{g/mL}$ (Ersoy, E. et al., 2019). But, Collagen/Chitosan/PHBV/*Hypericum perforatum* electrospun material showed no detectable inhibitory activity. This may be due to complexation of *H. perforatum* polyphenols with polysaccharides and proteins. Polyphenols which are responsible for collagenase inhibition might have been occupied by chitosan and collagen which are present in the blend (Popa et al., 2000).

IV. CONCLUDING RESULTS AND RECOMMENDATIONS

In this study, a nanofiber wound dressing was introduced for the first time consisting PHBV/Collagen/Chitosan and *Hypericum perforatum* extract by electrospinning method designed for wound dressing. Electrospinning being functional and simple technique with many biomedical applications has attracted the interest of researchers. Development of electrospun scaffolds for skin engineering seems to be in the primary level. Pure chitosan cannot be easily electrospun, so PHBV/Collagen was blended to improve the spinning process. As *Hypericum perforatum* extract added to enhance the healing capacity of the nanofiber. Hydrophilic dressing platforms with good mechanical properties active functional groups will assist in controlling the hydrolytic enzymatic activities at the wound site. Effective dressings optimized for wound healing must not be invasive have a reasonable cost. Biodegradable polymers with low cytotoxicity and permeability must be selected for wound dressing.

In our study we produced PHBV/Collagen and chitosan, based nanofiber and characterized it for further uses. We expect that this thesis will provide inspiration for researchers who are working in biomedical field.

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PUBLICATIONS AND PRESENTATIONS

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Development and characterization of bionanocomposites from electrospun PHBV/Collagen/Chitosan/*Hypericum perforatum* extract for potential wound healing application, presented at 3rd International Euroasian Conference on Biological and Chemical sciences (EuroasianBioChem2020) between 19-20 March 2020, Ankara, Turkey.

INTERNSHIP

Internship of three months between 03.09.2015 and 27.11.2015 at The Gabonese National Center of Blood Transfusion, in the laboratory of Immunology -hematology and virology (CNTS).