

T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF NANOTECHNOLOGY

**DEVELOPMENT OF FISH SCALE-DERIVED COLLAGEN AND
SODIUM ALGINATE COMPOSITE FILM STRUCTURES FOR
WOUND HEALING APPLICATIONS**

MELİKE KESKİN
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF NANOSCIENCE AND NANOENGINEERING

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THESIS SUPERVISOR
ASST. PROF. DR. OSMAN EKSİK

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T.R.
GEBZE TEKNİK ÜNİVERSİTESİ
NANOTEKNOLOJİ ENSTİTÜSÜ

YARA İYİLEŞTİRME UYGULAMALARI
İÇİN BALIK PULU TÜREVİ KOLLAJEN VE
SODYUM ALJİNAT KOMPOZİT FİLM
YAPILARININ GELİŞTİRİLMESİ

MELİKE KESKİN
YÜKSEK LİSANS TEZİ
NANOBİLİM VE NANOMÜHENDİSLİK

TEZ DANIŞMANI
DR. ÖĞR. ÜYESİ OSMAN EKSİK

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SUMMARY

The human skin, which is the body's biggest organ, is vulnerable to a variety of external causes that can cause injury. These external causes could potentially cause a delay in the wound healing process. Collagen activates fibroblasts, promoting the deposition of new collagen in wound beds. Alginate helps prevent bacterial infections in wound.

The purpose of this study is to harvest collagen from the scales of the *Dicentrarchus labrax* and then mix that harvested collagen with sodium alginate to generate a composite film that may be used in the treatment of wounds. In order to recycle the waste and reduce the cost, pepsin-solubilized collagen (PSC) was extracted from the fish scales of *Dicentrarchus labrax*, and then a composite film was formed using the solvent casting method with commercially available sodium alginate. These two stages make up the research that was carried out in accordance with this objective. The chemical composition, structural morphology, thermal stability, and elemental analysis of the films that were formed were characterized by using FTIR, SEM-EDS, DSC, TGA, XRD analyses.

As a result of the analysis, it was found that the first stage of the experimental process, collagen, was successfully acquired, and that the film form composited with sodium alginate in the second stage was effectively generated. Intermolecular connections were shown to be established between sodium alginate and collagen, as well as the elemental presence of both sodium alginate and collagen in the composite film. Our findings demonstrate that these film variations are promising in wound healing.

Keywords: Collagen, thermal stability, morphology, sea bass (*Dicentrarchus labrax*) scale, sodium alginate.

ÖZET

Vücudun en büyük organı olan insan derisi, yaralanmaya neden olabilecek çeşitli dış etkenlere karşı savunmasızdır. Bu dış etkenler potansiyel olarak yara iyileşme sürecinde bir gecikmeye neden olabilir. Kolajenin fibroblastları harekete geçirir, yara yataklarında yeni kollajenin birikimini teşvik eder. Aljinat, yaradaki bakteriyel enfeksiyonların önlenmesine yardımcı olur.

Bu çalışmanın amacı, *Dicentrarchus labrax* pullarından kolajen toplamak ve daha sonra bu toplanan kolajeni sodyum aljinat ile karıştırarak yaraların tedavisinde kullanılabilecek bir kompozit film oluşturmaktır. Atığın geri dönüştürülmesi ve maliyetin düşürülmesi amacıyla *Dicentrarchus labrax* pullarından pepsin ile çözündürülmüş kollajen (PSC) ekstrakte edilmiş ve daha sonra ticari olarak temin edilebilen sodyum aljinat ile çözücü döküm yöntemi kullanılarak bir kompozit film oluşturulmuştur. Bu amaç doğrultusunda yürütülen araştırmayı bu iki aşama oluşturmaktadır. Oluşan filmlerin kimyasal bileşimleri, yapısal morfolojileri, termal kararlılıkları ve elementel analizleri FTIR, SEM-EDS, DSC, TGA, XRD analizleri kullanılarak karakterize edilmiştir.

Yapılan analizler sonucunda deneysel sürecin ilk aşaması olan kolajenin başarılı bir şekilde elde edildiği, ikinci aşamada sodyum aljinat ile birleştirilen film formunun etkin bir şekilde oluşturulduğu görülmüştür. Kompozit filmde hem sodyum aljinat hem de kollajenin temel varlığının yanı sıra sodyum aljinat ve kollajen arasında moleküller arası bağlantıların kurulduğu gösterildi. Sonuçlarımıza göre, bu farklı film versiyonu yara iyileştirme alanında umut vaat etmektedir.

Anahtar Kelimeler: Kolajen, termal stabilite, morfoloji, levrek (*Dicentrarchus labrax*) pulu, sodyum aljinat.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
ACS:	Acid-soluble collagen
DSC:	Differential scanning calorimetry
ECM:	Extracellular matrix
EDS:	Energy dispersive spectroscopy
EDTA:	Ethylenediamine tetraacetic acid
FGF:	Fibroblast growth factor
FTIR:	Fourier-transform infrared spectroscopy
HCl:	Hydrochloric acid
NaOH:	Sodium hydroxide
PCS:	Pepsin-soluble collagen
PDGF:	Platelet-derived growth factor
PEF:	Pulsed electric field
SA:	Sodium alginate
SBE:	Semi-bionic extraction
SEM:	Scanning electron microscope
TGA:	Thermogravimetric analysis
UV:	Ultraviolet
VEGF:	Vascular endothelial growth factor
XRD:	X-Ray diffraction

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1. INTRODUCTION

Every day, individuals sustain a variety of injuries to their skin, including deep lacerations as well as more superficial wounds that are the result of abrasions, cuts, and mild burns [1]. In most cases, the patients themselves will apply first aid dressings to these relatively minor wounds in order to treat them [2]. These dressings provide a barrier that protects the wound from debris, bacterial contamination, and mechanical forces like pressure and friction [2]. However, the typical practice of applying wound dressings at home often results in a dry wound environment [3]. According to Dauntton *et al.*, the process by which an acute wound heals in humans and animals is a highly sophisticated event that involves three phases: the hemostasis/inflammatory phase, the proliferation phase, and the remodeling phase [4]. The expression of dermal enzymes such as hyaluronidase, elastase, and collagenase (matrix metalloproteinases-1) was elevated as a consequence of the inflammatory response. Hyaluronic acid, elastin, and collagen of the extracellular matrix are hydrolyzed during this process. According to Boran *et al.*, this mechanism could play a significant part in the formation of wounds on the skin [5].

Since ancient times, many methods have been used to heal wounds, and the majority of these methods typically entail the use of gauze or other materials to dress and bandage wounds [4]. Some of these methods date back even farther than ancient times. However, removal of such dressings may cause harm to the area that is healing [4]. This is due to the fact that gauze has a tendency to adhere to the site and may leave fibers and debris behind [6]. Because of this, more suitable dressings must have been devised in order to compensate for these deficiencies [6]. Even though gauze has been shown to be helpful in a variety of circumstances, medical professionals and other health care workers have a responsibility to be aware that this material is not ideal and that it does not comply with the standards against wound damage when it is used [7]. According to Sood *et al.*, woven gauze inhibits the healing process of wounds since it must be removed with force [7].

In the past several years, biopolymers and biochemicals have been used in combinations as perfect candidates for novel wound dressing materials due to their superior properties [8]. These superior properties include the capability to maintain a non-traumatic, non-adherent, non-toxic and moist wound environment, as well as favoring antibiotic and gas permeability while preventing maceration of the

surrounding tissue [9]. On the market, one can find many different types of biopolymer wound dressings, each of which has the role of maintaining wound hydration and optimizing skin regeneration, protecting against bacterial infection, and preventing disturbance of the wound base [10]. These biopolymer wound dressings also have the ability to protect against bacterial infection [10]. In the research and development of wound dressings and other wound healing materials, collagen and sodium alginate are two of the biomaterials that are used most commonly.

According to Friess *et al.*, more than thirty distinct types of collagen have been isolated from a variety of animal species [11]. Collagen is one of the most prevalent proteins discovered in the animal world [12]. After a skin injury, collagens contribute to the local storage and transport of growth factors and cytokines, which is a process that occurs throughout organ development [12]. Because of their importance in tissue regeneration and wound healing, as well as the fact that collagen is a common ingredient in wound dressings [13].

A variety of wound healing effects may be attributed to sodium alginate as a result of its singular features and interactions with the milieu of the wound [14]. When applied to wounds in the form of a dressing, sodium alginate has the potential to hasten the healing process and enhance the overall prognosis for the patient [15]. Its capacity to generate a moist environment, allow autolytic debridement, and build a protective gel matrix all contribute to an enhanced wound healing process, which in turn decreases the likelihood of infection and perhaps improves patient comfort and aesthetic results [16]. However, in order for wound care to be successful, a thorough evaluation of the particular patient's requirements and consideration of any medical interventions that may be necessary are essential [15].

In order to develop a sustainable new wound healing material, the purpose of this thesis study was to extract collagen from discarded sea bass scale (*Dicentrarchus labrax*) and to construct a composite film using commercially available sodium alginate. This study was carried out in accordance with this aim and consists of two stages: first pepsin-solubilized type I collagen (PSC) was extracted from sea bass fish (*Dicentrarchus labrax*) scales in order to recycle waste and reduce the cost of wound dressing materials. Next, a composite film was formed with commercially available sodium alginate using the solvent casting method.

The following topics are covered in this thesis: skin, wound, wound types, wound healing process, collagen, collagen structure, collagen types, function of

different types of collagens, Natural Polymers Used in Wound Healing, collagen and sodium alginate. After this, there is a section called the literature review in which current research on wound healing that have been published in the literature are analyzed. The third section consists of experimental research, and then the fourth part is the results and the discussion of those studies. The overall assessment of the research was carried out in the last section, which was followed by the drawing of the conclusion.

1.1. Skin

The skin produces secretions that are capable of killing bacteria, and the pigment melanin offers a chemical defense against UV radiation, which is known to harm skin cells [17]. It plays a vital function in preserving homeostasis, by controlling body temperature, halting water loss, and guarding against dangerous microorganisms and ultraviolet (UV) radiation [18]. Hemostasis which refers to the capacity of the organism to keep its internal environment at a constant temperature and pressure is essential to keep homeostasis going in order to provide a favorable environment in which wound healing may take place [19]. As seen in the Figure 1.1., the skin is comprised of three essential layers: the epidermis, dermis, and hypodermis [20]. The epidermis is the skin's top layer and is composed of keratinocytes, melanocytes, and Langerhans cells in addition to other layers of cells. Keratin gives the skin its waterproofing and its strength, both of which are necessary during the early phases of wound healing to avoid additional damage and infection [17]. Melanocytes generate melanin, a pigment that gives color to the skin and protects against UV radiation [19]. Wounds that are in the process of healing might be negatively affected by UV light, which can either delay the process down or lead to problems. During the process of wound healing, the presence of melanin in the skin serves as an extra barrier of protection.

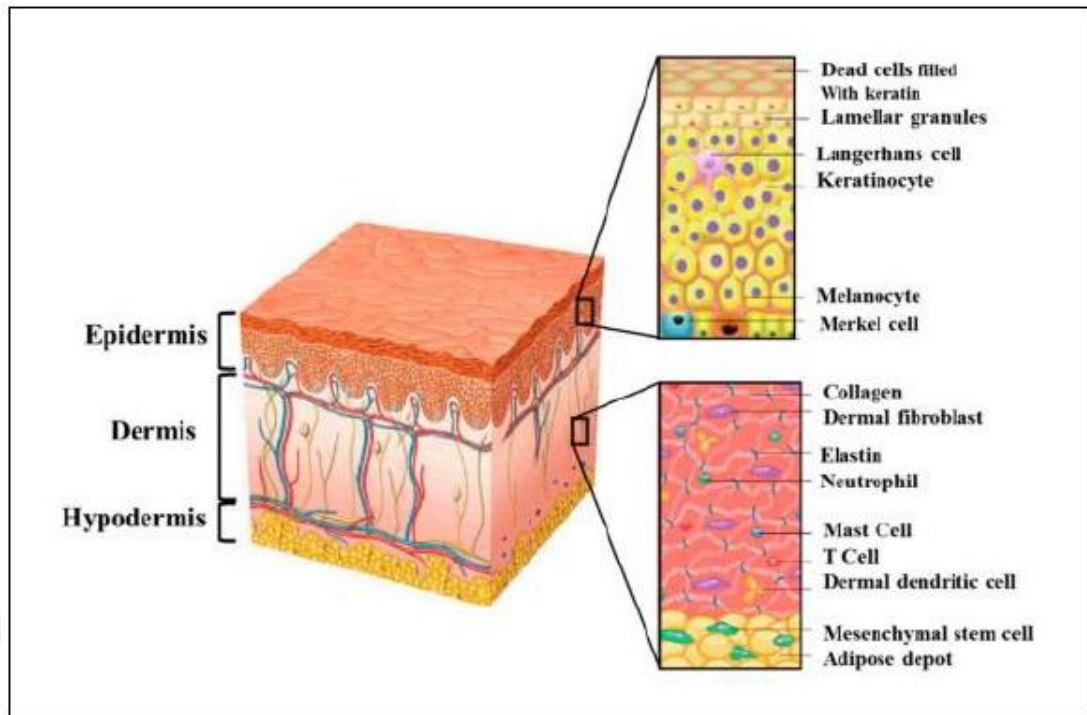


Figure 1.1: The essential layers of the skin is epidermis, dermis and hypodermis [20].

Beneath the epidermis is the dermis, which contains blood vessels, nerves, hair follicles, and sweat glands. The dermis includes collagen and elastin fibers, that give the skin its strength and elasticity, and it supports the skin structurally. The dermis also houses immune cells that help defend against infections [21]. The innermost layer of skin is called the hypodermis, sometimes referred to as the subcutaneous tissue. It arises from fat cells that insulate and cushion the body. Blood arteries that supply the hypodermis with nourishment are also present [22].

The skin is constantly renewing itself through a process called cell turnover. In the epidermis' basal layer, brand-new cells are created and gradually proliferate until shedding off at the surface. The functionality and integrity of the skin are preserved by this process [23]. The capacity of the skin to continually renew itself via the process of cell turnover is essential for the healing of wounds [24]. When an injury occurs to the skin, the body immediately begins the process of repairing the damage by initiating a healing process that includes the proliferation of new cells and their migration to the location of the wound [25]. A substantial part of this process is governed by the epidermis' basal layer, which is the location where new skin cells are generated [25].

The ongoing generation of new cells guarantees a consistent supply of cells that may be used for the healing and closure of wounds [24].

The skin serves a protective purpose, but it also aids in sensory awareness [26]. It comprises innumerable sensory receptors that determine pain, temperature and touch [26]. These receptors send signals to the brain, permitting us to sense and respond to our environment [27]. These receptors are absolutely necessary for determining whether or not a wound harbors any possible dangers or sources of infection [28]. When a person's skin is cut, the sensory receptors in that area quickly transmit messages to the brain, therefore warning the person about the wound and any possible threats that it may provide [29]. This motivates protective actions, such as avoiding additional harm or seeking proper medical treatment, both of which are critical for ensuring the wound heals without problems and ensuring that protective behaviors are undertaken [28].

When the skin is healthy, it acts as a barrier against pathogenic bacteria and stops the body from losing an excessive amount of water [30]. Nevertheless, a breach in the skin's protective barrier occurs whenever the skin is injured, leaving the body open to the possibility of infection [31]. During the early phases of wound healing, the protective features of the skin are very important [30]. These properties help establish an environment that is conducive to the immunological response of the body, which helps clean away debris and fight off possible infections [32].

In general, the skin is a dynamic and sophisticated organ that serves a variety of purposes to preserve the body's health and wellbeing [33]. For the diagnosis and treatment of numerous skin problems and diseases including wounds, it is crucial to comprehend the structure and function of the skin [34]. In order to successfully evaluate the severity of wounds, detect possible complications, and establish treatment plans that are suitable, experts in the healthcare industry need to be familiar with the natural processes and properties of the skin [35]. In addition, having a grasp of the structure of the skin makes it easier to choose proper wound treatment that will promote and speed the process of wound healing [30].

1.2. Wound

The anatomical structure which consists the layers of the skin as illustrated in Figure 1.1 and function of the skin deteriorate or are damaged as a result of the wound [36]. As a result of simple or deeper deterioration of the epithelial structure of the skin, nerves, tendons, muscles or bones can be destructed [37]. Wounds can occur in a healthy individual as a result of any external intervention such as burns, abrasions, surgical procedures, crush injuries, cuts and lacerations, or as a result of a disease such as diabetes mellitus, cancer, peripheral arterial disease, vasculitis [38]. The creation of a wound triggers a series of physiological responses in the body, including bleeding, coagulation, vascular contraction, activation of complement, and inflammatory response [38].

After wound formation, the healing process begins immediately, and in this process, cell migration includes the effect of the extracellular matrix. The following is the structure of the mechanism that underlies the process from the point of wound creation to the point of healing: (I) inflammatory mediators, (II) cell to cell and cell to extracellular matrix (ECM) interactions, (III) epithelialization, fibroplasia and angiogenesis procedures, (IV) contraction of wound, (V) step of remodeling [39]. The anatomical and physiological differences of structures such as skeletal tissue, liver and eye cause differentiation in the remodelling and renovation processes in the wound healing continuum [40]. There is a possibility that the duration of the regeneration process may change depending on the tissue [41]. The tissue eventually returns to its usual look and anatomical structure once the wound healing process has been completed and has taken some time. The natural wound healing continuum can be altered and made chronic by the variety of local and systemic agents [42]. Even while the natural process of wound healing is a very efficient and intricate system, it does have a few restrictions, and some situations or causes may impede its capacity to accomplish full healing [43]. The following is a list of some of the therapeutic limitations of the natural wound healing process: a lack of proper diet, illness, the presence of foreign substances or detritus, advanced age, and impaired immune function [44]. The majority of cuts and scrapes that aren't very serious may heal on their own without the assistance of any external wound healing products or procedures [45]. Wounds such as tiny cuts and scrapes, minor burns, bruises, small blisters, and

insect bites and stings are all examples of wounds that may normally heal on their own without the need for the use of wound healing materials [46].

1.2.1. Type of Wounds

Wounds are able to be classed depending on a variety of variables, such as the origin of the wound, the depth of the wound, the physical appearance of the wound, and the chronicity of the wound [47]. While there are many different ways to categorize wounds, the length of time it takes for them to heal determines whether they are acute or chronic [36]. When it comes to providing patients with the highest level of care and enhancing their overall results, accurate wound categorization is very necessary [48]. It is essential to point out that wound evaluation and classification are tasks that must be carried out by trained medical experts in order to guarantee an accurate diagnosis and effective treatment planning [49].

1.2.1.1.Acute Wounds

These are the types of wounds in which the healing process progresses as it should, and heal functionally and anatomically in a regular and timely manner. While the healing period generally takes 5-10 days contingent upon the depth of the wound, there are also wounds that last 30 days. Acute wounds can originate from traumatic tissue loss or after a surgical operation [38]. The formation of acute wounds as a result of surgical operation is common.

Contrarily, traumatic wounds may originate from soft tissue injury and bone fractures and may result in a combination of injuries. The AO Foundation's categorization system (Arbeitsgemeinschaft fuer Osteosynthesefragen/Association for the Study of Internal Fixation) is used to categorize these injuries into several species. [50]. One benefit of this classification method is that the prognosis of the wounded area can be assessed by taking into account how much the muscles and tendons have been damaged [50]. The application of direct pressure with a clean cloth or sterile bandage is the initial treatment stage for acute traumatic wounds [51]. This helps to stop bleeding and is the first step in the therapy process. It's possible that simple adhesive bandages or dressings are all that's needed to treat small wounds [52].

Dressings that are more technologically sophisticated, such as hydrocolloids, films, or foams, may be utilized on wounds that are wider or deeper [51]. Healthcare practitioners may use sutures (stitches), staples, or adhesive strips to seal the open margins and encourage appropriate wound healing [44]. This is especially helpful for wounds that are very deep or gaping [52]. This assists in bringing the wound edges together and lowers the amount of scarring that occurs as a result [53]. It is possible to avoid infection by using topical antiseptic creams or solutions to wounds that have been well cleaned [53].

1.2.1.2. Chronic Wounds

Chronic wounds are defined as ones that do not make progress through the regular phases of healing, and they are unable to be mended in an organized and timely way either [54]. The process of healing is incomplete and is impeded by a variety of circumstances, which extend one or more stages within the phases of haemostasis, inflammation, proliferation, or remodelling [55]. Tissue hypoxia, infection, exudate, necrosis and excessive inflammatory cytokine levels can be counted among these factors [56]. Persistent inflammation brings with it non-healing scar tissue. Since the healing process is interrupted, it disrupts the functional and anatomical structure and this causes the recurrence of wounds [56]. Factors such as pressure, arterial and venous insufficiency, vasculitis and burns are factors that can cause chronic wounds [55].

It is crucial to grasp the connection between chronic wounds and the defective phases of hemostasis, inflammation, proliferation, or remodeling in order to comprehend why chronic wounds do not heal and why they may repeat [53]. Chronic wounds are wounds that do not go through the regular phases of wound healing within the predicted period (which is normally between four and six weeks) [57]. Chronic wounds may last for months or even years [57]. It is possible for them to continue to be in an inflammatory condition for an extended period of time or to show signs of delayed or stopped recovery [58]. The disruption of the normal wound healing cascade caused by a number of different causes may be blamed for the slowed down healing process [58].

1.2.1.3. Complex Wounds

Wounds that come with a variety of problems that interfere with the usual healing process are referred to as complex wounds [59]. Because of the many variables that might slow or impede the healing process, these wounds often need for specific medical attention and treatment [60]. Delay in healing, chronicity, underlying health conditions, infection, necrotic tissue, and vascular issues are some of the signs of complex wounds [61]. To explain these signs in detail, the following can be said: Complex wounds often take far longer than anticipated to heal, during which time they frequently stay in the inflammatory phase of the wound healing process [62]. Because they do not move through the regular phases of wound healing within the predicted period (which is normally between four and six weeks), complex wounds are sometimes categorized as chronic wounds [63]. Diabetes, peripheral vascular disease, venous insufficiency, autoimmune diseases, and malnutrition are all examples of underlying health issues that might hinder the body's capacity to heal complicated wounds [63]. Malnutrition can also contribute to the development of complex wounds [62]. Because of the presence of foreign material, necrotic tissue, or a defective immune response, complex wounds have a much increased likelihood of getting infected [64]. The presence of non-viable or dead tissue in the wound might slow down the healing process and create an environment that is conducive to the development of germs [64]. Complex wounds are more likely to develop from simple wounds if they are situated in regions of the body that have an inadequate blood supply. This includes the lower extremities [65].

1.3. Process of Wound Healing

The body's reaction to a destruction sustained by a tissue is the beginning of a series of complicated and ever-changing processes known as the wound healing process [66]. The wound healing continuum is the same for all wounds. The healing process is a process in which various chemical and immunological events follow each other [44]. In order to restore the integrity of the skin and repair the injured tissue, the healing process is broken down into many stages [67]. The total amount of time it takes

for a wound to heal may vary based on the kind of wound, its size, its depth, and the individual's general health [68].

There are four stages in the acute, chronic and complex wound healing process: (I) Coagulation and hemostasis development (II) Quickly following, inflammation occurs (III) Proliferation process emerges a few days after wound formation and starts healing (IV) The healing process, which includes the creation of scars [40].

1.3.1. Coagulation and Hemostasis Stage

The first reaction of the body to a wound is called coagulation and hemostasis, and it includes the development of a blood clot in order to halt the bleeding [69]. At this stage, it is aimed to prevent bleeding by protecting the vascular system and preventing its deformation. Thus, no functional damage occurs in vital organs. The long-term goal is to build a matrix for the invasive cells participating in the healing process' final stages [70]. Hemostasis is controlled by the dynamic equilibrium between platelets, coagulation, endothelial cells, and fibrinolysis. Fibrin deposition at the location of the wound is then determined by this balance, which impacts how quickly the repair process moves forward [50].

As a result of attack and microvascular injury, blood can leak into the wound [67]. Any injury to the vessels causes contraction of these vessels as a result of the reflex mechanism involved. Thanks to this contraction, bleeding can be strongly prevented. This process develops only in vessels with transverse interrupted.

Conversely, it causes an increase in wound patency in interrupted arterioles [71]. Reflexive vasoconstriction reduces bleeding for a few minutes. If this reflexive process did not develop, the hemostatic mechanism produced by the body may become ineffective for long periods [42].

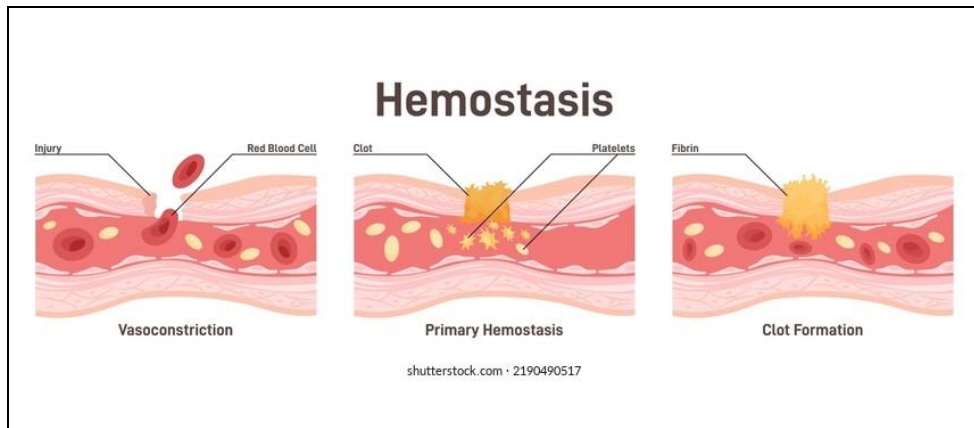


Figure 1.2: A illustration in schematic form of the biological process of hemostasis [72].

As seen in the Figure 1.2, platelet aggregation is activated by various extrinsic and intrinsic pathways and the coagulation process begins [72]. Blood formed at the site of injury comes into contact with extracellular matrix components such as platelets and collagen. The sending of clotting factors from platelets and the development of blood clots are triggered at this precise instant of contact [73]. During the healing process, the clot's surroundings serves as a temporary matrix environment for cell migration. Growth factors, insulin-like growth factors, epidermal growth factor, platelet-derived growth factor (PDGF) are found in the cytoplasm of platelets [74]. These growth factors activate neutrophils, macrophages, fibroblasts, and endothelial cells, which aid in the promotion of wound healing [74]. Serotonin and other vasoactive amines found in platelets induce fluid to extravasate, forming edema in the tissue. The inflammatory phase of this edema causes it to become stronger [70]. When a cell membrane is injured, metabolic byproducts of arachidonic acid are produced, and they have biological effects that cause an inflammatory reaction [75].

The creation of a blood clot as a protective mechanism in response to a damage to tissue is an essential process that paves the way for future phases of wound healing [70]. The clot acts as a foundation for the inflow of cells and growth factors that are essential for the repair and regeneration of the injured tissue [66]. This is accomplished via the process known as neovascularization [66]. The blood clot will eventually be replaced by granulation tissue and new tissue creation as the wound healing process continues [67]. This will finally result in the wound being sealed and its original state being restored [76].

1.3.2. Inflammatory Phase

The cellular and humoral inflammatory phases follow to build an immune defense against encroaching pathogens. The early and late inflammatory phase are the two separate phases [77]. In the process of wound healing, the inflammatory phase is an important step that is vital to its progression [78]. This stage creates a beneficial environment for the continuation of the healing process such as proliferation and remodeling which is schematized in Figure 1.3, and lays the groundwork for the next stages of the recovery process [79]. To guarantee good wound regeneration without excessive scarring or the formation of chronic wounds, it is essential to properly regulate the inflammation and bring it under control as quickly as possible [80]. The inflammatory phase is illustrative of the complexities that are involved in the natural healing processes of the body [77]. It also highlights the significance of a well-balanced and well-coordinated immune response for the purpose of achieving optimum wound healing [80].

The features of the collagen and sodium alginate composite film may promote and improve the inflammatory response that occurs during the process of wound healing [81]. The capability of the film to both provide a moist environment and encourage the regeneration of damaged tissue may facilitate the movement and activity of immune cells, such as neutrophils and macrophages, at the site of the wound [82]. In addition, the presence of collagen in the film may contribute to the release of growth factors, which are important for wound healing since they promote cellular processes [83].

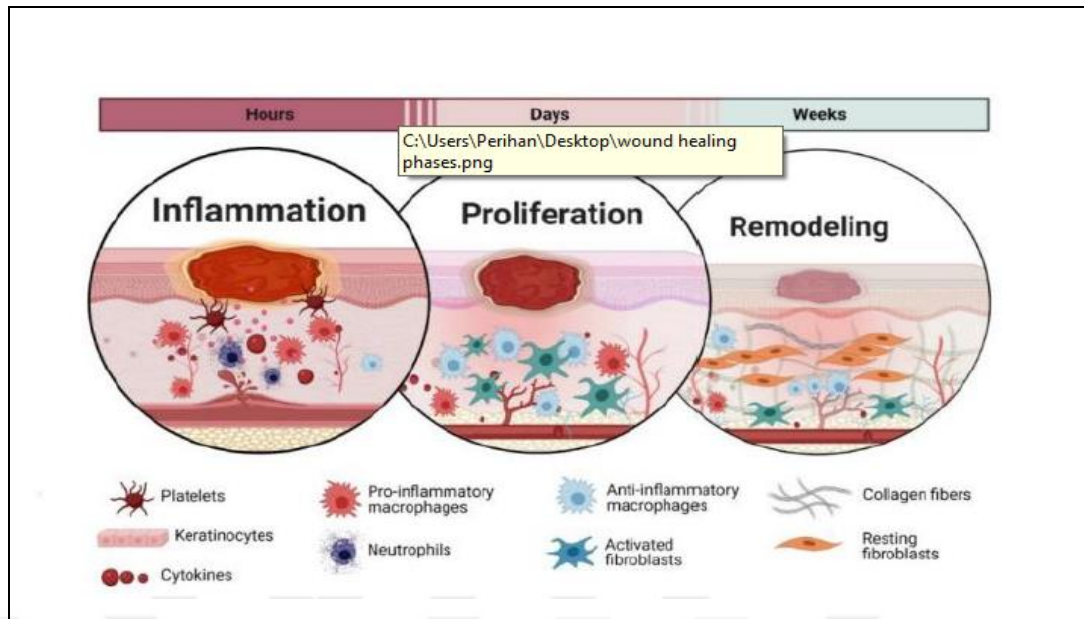


Figure 1.3: The inflammation, proliferation and remodeling steps of wound healing [79].

1.3.2.1. Early Inflammatory Phase

Several functions are served by the early inflammatory response, which begins in the late coagulation phase. It starts the molecular processes that let neutrophils move toward the wound and activates the complement chain [67]. To remove bacteria, foreign particles and injured tissue, phagocytosis by neutrophils is their first and most important task. Phagocytotic activity is of great importance for the healing of acute wounds with bacterial imbalance [84].

The activation of numerous chemoattractive factors causes neutrophils to begin migrating to the wound site 24 to 36 hours after the lesion first appears. Neutrophils transform into sticky particles and cling to endothelial cells surrounding the lesion as a result of the rearrangement of adhesion molecules [84]. It then travels along the endothelial surface with the blood flow. Weak bonds develop due to adhesion and various mechanisms [40]. Strengthening of the adhesion system is activated through cellular activities [41]. Cells halt moving and immigrate out of the venules, becoming trapped between endothelial cells [41]. Neutrophils destroy bacteria by releasing free radical species [85]. After the bacteria are destroyed, the neutrophils are eliminated.

Excess cells are admittedly shed to the wound surface by apoptosis and extrusion [40]. Cell remnants are phagocytosed by macrophages [68].

The collagen and sodium alginate composite film that we developed may interact with inflammatory cells, cytokines, and growth factors that are present at the wound site [86]. Therefore, it may help to promote an appropriate level of inflammation. Once coupled with water or wound exudate, the sodium alginate that is included in the film is able to provide a moist environment at the wound site [87]. The presence of moisture in the surrounding environment encourages the movement of immune cells to the injured location [88]. The immune cells and fibroblasts that are present during the early inflammatory phase are essential for the healing of tissue [88]. It is possible that the collagen and sodium alginate composite film will encourage the migration and multiplication of these cells [89]. Studies have been conducted on sodium alginate to investigate the possibility that it has antibacterial qualities [90]. If shown to possess such capabilities, sodium alginate would be able to assist lower the risk of infection at the wound site [91]. Because infection may cause inflammation to last for a longer period of time and slow the healing process, it may be useful to have access to a substance that can inhibit the development of bacteria [91].

1.3.2.2. Late Inflammatory Phase

Within forty eight - seventy two hours of the formation of the wound, macrophages start to develop in the area and carry on the phagocytosis process [76]. Macrophages are blood monocytes, undergo phenotypic change when they enter wounded tissue and are drawn to the wound site by chemoattractive substances such as cytokines, elastin, and collagen degradation products. Compared to neutrophils, macrophages can survive longer and can continue to occupation at lower pH levels [92]. These cells activate various growth factors, fibroblasts and endothelial cells [92]. Depletion of macrophages and monocytes from the wound site results in delayed fibroblast proliferation, weakened wound debride, and severe defects in healing, resulting in poor wound repair [93]. After 72 hours, lymphocyte cells which is the type of white blood cell, are the last to reach the wound site [91]. Lymphocytes are responsible for the production of a variety of cytokines and growth factors that promote the activity of fibroblasts, which in turn results in the synthesis and

organization of collagen [80]. It does so by secreting substances that stimulate angiogenesis and guarantee a sufficient blood supply, both of which are necessary to enable tissue healing [91].

1.3.3. Proliferative Phase

Once the wound has hemostasis and the immune system responds, the wound begins tissue repair [84]. The proliferative phasis of repair, that starts on the third day following the injury and typically lasts for two weeks, is at this stage. A stage known for the buildup of recently generated ECM and fibroblast migration which begin the process of producing new collagen fibers and depositing them there [94]. In this phase, the formation of granulation tissue is observed.

During this stage, the goal moves from managing inflammation to repairing and regenerating broken tissue as the primary emphasis [95]. This extracellular matrix acts as a base for the migration of the many cell types that are involved in the healing of tissue [96]. These collagen fibers aid in the healing process by providing support to the wound and stimulating new tissue growth [97]. Granulation tissue is a characteristic that may be identified with the proliferative phase [98]. It is a thin tissue that is pinkish in color and granular in appearance. It is made up of newly formed blood vessels (angiogenesis), fibroblasts, and a variety of ECM components. Granulation tissue contributes to the process of filling the wound area and establishing a supporting bed for the regeneration of more tissue [99]. Below are descriptions of each process that takes place during the proliferative phase.

1.3.3.1. Fibroblast Migration

After the wound is formed, myofibroblasts and fibroblasts in that area are stimulated to proliferate for three days [93]. They then move into the wound while being assisted by a number of growth factors produced by platelets and inflammatory cells [88]. Phenotypic modulation is necessary for fibroblast accumulations that occur on the third day. They create matrix proteins, hyaluronan, proteoglycans, type 1 and type 3 procollagen, fibronectin after proliferating at the site of the wound [92].

Abundant extracellular matrix deposition occurs seven days after wound formation [92]. Fibroblasts transform into myofibroblast phenotypes, and in this transformation stage, the thick actin bundles they contain under the plasma membrane bind to collagen and fibronectin in the ECM and lengthen the pseudopodia [88]. Wound contraction, which supports the convergence of the wound edges, occurs by retraction of the cell extensions. At the end of this process, fibroblasts that complete their task are eliminated by apoptosis [44].

1.3.3.2. Collagen Synthesis

They are synthesized by collagen fibroblasts and affect all stages of wound healing, especially the proliferative and remodeling stages [100]. They contribute to the creation of the intracellular matrix in the wound and give the tissues strength and integrity. Collagen types 1 and 3 build up 80% and 25%, respectively, of the healthy dermis tissue, and 40% and 40%, respectively, of the granulation tissue [101]. This thesis study's first stage, which consisted of obtaining type I collagen from sea bass (*Dicentrarchus labrax*) scale and forming it into a composite film using sodium alginate. In this regard, it has been created to prevent the loss of type I collagen, which is the primary component of scar tissue. When a film composed of collagen type I and sodium alginate is put to When a film composed of collagen type I and sodium alginate is put to a wound, it will encourage the regeneration of damaged tissue and produce an environment that is conducive to the attachment, migration, and proliferation of cells. During the process of wound healing, the collagen type I film will also alter the distribution of collagen types 1 and 3, so contributing to the creation of an environment that is more conducive to the repair of damaged tissue.

1.3.3.3. Angiogenesis and Granulation Tissue Formation

All phases of wound healing simultaneously experience the creation of blood vessels, a crucial step in the healing process. During the process of wound healing, the formation of new blood vessels is critical to the delivery of nutrients and oxygen, the elimination of waste products, the support of the immune response, and the promotion of cell migration and proliferation [102]. The proliferation of new blood vessels

provides the many cells engaged in tissue regeneration with the nutrition, oxygen, and vital growth factors that are necessary for their continued survival and function [103]. At the location of the wound, the sodium alginate that is present in the composite film may be able to contribute to the preservation of a moist environment. Because new blood vessels prefer to develop in a wet environment, this is excellent for stimulating angiogenesis, which is the formation of new blood vessels. Blood vessels play an important role in the removal of waste products and cellular debris from the wound site, which in turn helps to facilitate the clearing of dead tissue and promotes an environment that is clean and conducive to the healing process [104]. At the location of the wound, the influx of immune cells that occurs as a result of the formation of new blood vessels both aids in the fight against infection and strengthens the immune response [104]. The development of new blood vessels is absolutely necessary for the migration and proliferation of cells like fibroblasts and epithelial cells, which both play important roles in the process of tissue regeneration [103]. Angiogenic factors attract neutrophils and macrophages, while at the same time stimulating the angiogenesis process [105].

It is possible that the characteristics of the collagen and sodium alginate composite film will serve to manage the inflammatory response that occurs during the process of wound healing. This will help to ensure that the inflammation is regulated and balanced, which is essential for the development of granulation tissue and angiogenesis. The collagen and sodium alginate composite film has the potential to serve as a temporary scaffold for the creation of granulation tissue. It offers a three-dimensional framework that makes it easier for cells, such as fibroblasts and endothelial cells, to migrate into the area of the wound where they are needed. It is feasible for collagen, which is utilized in this thesis study as an important component of the extracellular matrix, to induce the attachment of cells, migration of cells, and regeneration of tissue. Collagen is used in this thesis study as an essential component of the extracellular matrix.

Endothelial cells react to many angiogenic factors, especially various growth factors. Mitosis is triggered by stimuli and inhibitors, which promote host cell migration and the production of growth factors [106]. The tissue around the wound provides the molecules required for the development and proliferation of endothelial cells. In response, the steps of the development process are as follows: (I) generation of proteases by endothelial cells to breakdown the basal lamina in the main vessel, (II)

chemotaxis, (III) proliferation, and (IV) remodelin. Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF), which are growth factors, act as regulators in this whole process [107]. The surrounding live tissue is perfused by diffusion from the unwounded interstitium throughout the primary phase of wound formation because the wound center lacks vascular nourishment [108]. A microvascular network is created in the wounded location as capillaries from the surrounding healthy tissue migrate there.

Chemotaxis, the process by which cells move along a chemical gradient, enables cells to proliferate, differentiate, and respond to environmental stimuli [109]. These agents act on the receptors throughout the wound healing process and direct the cell migration that develops in the angiogenesis phase [110]. Contributing factors are mediators involved in the process of vascular repair and neovascularization in the wound. These factors are also effective in cell differentiation and growth [111]. Migration, which is the result of chemotactic activity, involves the organization of the cytoskeleton, cell adhesion, and signal transduction. Several chemical and physical agents operating in the vascular system control migration [112]. Three factors are effective in providing regulation, these factors are as follows: (I) chemotaxis, (II) mechanotaxis, (III) haptotaxis [113].

The three distinct mechanisms necessary for cell motility are cell front protrusion, adhesion to connect the actin cytoskeleton to the substrate, and traction to move the posterior cytoplasm onward [114].

1.3.3.4. Protrusion

Cell-cell adhesions and cell-ECM adhesions serve as anchor points for the cytoskeleton, which has three different types of connected filaments and supports the cell mechanically [112]. With its dynamic structure, the actin network is of great importance for organizing cell migration. Actin polymerization occurs near the leading side, which is where the concentration of chemoattractants is highest. They cause the formation of a protruding structure, that is stuffed with filamentous actin [115]. Actin filaments in the front and back of the leading edge assemble and disassemble to maintain the motion of the cell [116]. The morphological structure of the cell is controlled by regulatory protein and multiple signaling pathways [117].

In a manner similar to that in which they interact with the natural extracellular matrix, cells, when they come into contact with a collagen and sodium alginate film, are able to create adhesions with the surface of the film. Cells are able to attach themselves, disseminate out, and migrate because to the film's ability to offer anchor sites for cell-ECM adhesions. When it comes to arranging cell movement on the surface of the film, the dynamic topology of the actin network is very essential.

1.3.3.5. Adhesion

Integrins act as primary receptors for cell motility and are of great importance in the process of cell migration [118]. As a result of this process, adhesion occurs [117]. As the adhesion increases, the migration process accelerates, but the mobility decreases inversely with the attachment [118]. Weakly adhesive cells and endothelial cells can move faster and adjust adhesion density than cells with high adhesive forces. It binds to the extracellular matrix and changes the oval shape of the cell and turns it into a flat structure. These changes in morphology evolve under the management of integrin signaling [118]. Endothelial cells are the cell type that provides the fastest migration after injury [119].

1.3.3.6. Traction

The contractile force resulting from the connection between the integrin and the cytoskeleton causes the cell to pull on the substrate and pull the cytoplasm forward [112]. This force is provided by myosin motor proteins. An interaction occurs between the active fibers and myosin, and this interaction causes the cell body to be pulled anteriorly. Various chemical and physical stimuli determine the direction of cell migration [120]. The density of the blood vessels in the wound area reduces as collagen builds up there, and eventually the granulation tissue matures into a scar [115].

1.3.3.7. Epithelialization

One or two hours after wound formation, epithelial cells migrate towards the scar tissue. While the cells first accumulate in a single layer, they multiply over time and form the basement membrane [100].

1.3.4. Remodelling Phase

During remodeling phase, the wound undergoes structural changes and maturation to restore normal tissue function [44]. Collagen is reorganized and remodelled during the remodeling phase, which is an important step in maintaining the strength and integrity of the wound [96]. The first arrangement is not nearly as sturdy as the well-organized collagen fibers that are present in healthy tissues [121]. During the remodeling phase, the body keeps altering and reorganizing the collagen fibers in the connective tissue [122]. Collagen fibers, which are initially arranged in a disorganized fashion, progressively take on a structure that is more ordered and parallel as time passes [121]. Throughout the proliferative phase, fibroblasts initially accumulate immature Type III collagen. The majority of the wound's fracture strength, however, is acquired throughout the remodeling phase, when Type III collagen is switched out for Type I collagen by matrix metalloproteinase activity. Cross-linking, rearrangement, and enhanced collagen packing are also part of this phase [96].

Another crucial activity that takes place throughout the remodeling stage is angiogenesis, the development of new blood vessels. It supports and intersects with other proliferative activities in the wound, providing oxygen and nutrients necessary for tissue regeneration [96]. The ECM also contributes significantly to the proliferative phase by offering physical and mechanical support for cell adhesion, proliferation, and migration. For optimum wound healing, ECM remodeling, which involves a balance between creation, degradation, and maturation, is essential [123].

This stage is a dynamic process that can be altered by a number of variables. Factors like infection, age, diabetes can interfere with the proper sequence and timing of the phases, leading to impaired wound healing [38]. Mechanical factors, such as stretch, can also affect the remodeling phase by promoting cell migration and inducing chromatin remodeling-mediated nuclear morphology changes [124].

1.4. Natural Polymers Used in Wound Healing

The majority of natural polymers are capable of biodegradation or bioabsorption and are compatible with living systems [125]. Chitosan, keratin and sodium alginate are the examples of those that exhibit anti-inflammatory and anti-microbial characteristics respectively [125]. As a result, natural proteins (such as keratin, casein, collagen, and silk) and polysaccharides (such as chitosan, alginate, and hyaluronic acid) have received increased interest in the production of wound dressings [126]. This is because of their similarity to macromolecules, which are recognized by the human body, thereby achieving ECM biomimicry [126].

1.4.1. Chitosan

Chitosan is one of the ligands that is utilized the most often. It has been said that it has a positive charge and has the ability to facilitate the better absorption of metal anion medications [127]. Because of the cationic charge that chitosan has, it has been used as a coating layer on negatively charged particle surfaces via the process of electrostatic adsorption [128]. Chitosan has also shown that it has the capacity to modulate the behavior of medication release [129]. The nanoparticles that have a coating of positively charged chitosan attract the negatively charged cell membrane, which increases the amount of weakly water-soluble medicines that are able to pass through the membrane [130].

Chitosan is a natural cationic polymer that is widely used in the manufacturing of hydrogels, membranes, and electrospun nanofibers for wound healing due to its remarkable diversity in terms of its functional applications [126]. In addition, because of the antibacterial qualities of chitosan dressings, these dressings have been chosen for the treatment of wounded tissue [131]. Hydrogels made of chitosan and alginate were created and then loaded with hesperidin in a variety of concentrations [132]. These hydrogel materials were tested in a mouse model with a full-thickness cutaneous wound in order to assess their effectiveness. According to the findings, the wound closure rate of the chitosan/alginate hydrogels was significantly higher than the rate of the gauze-treated wounds in the control group [132]. In a separate piece of research, a nanofiber mat containing chitosan, polyvinyl alcohol (PVA), and zinc oxide (ZnO)

was shown to have significant levels of antibacterial and antioxidant activity, pointing to the possibility that it might hasten the healing process of diabetic wounds [128].

1.4.2. Keratin

Keratin is one of the most prevalent types of protein that may be found in human or animal components, such as hair, wool, nails, and feathers. Epithelial keratins and trichocyte keratins are the two distinct types of keratins that may be distinguished from one another [133]. Epithelial keratins are soft keratins that include low levels of sulfur (less than one percent) and cysteine (less than three percent) [134]. They are the primary constituent of the stratum corneum of the skin and play an essential part in the process of maintaining the integrity of the epidermal cells. Trichocyte keratins are considered hard keratins because they include a greater amount of sulfur (5%) and cysteine (4%–17%). In the regions that link to the epidermis, they perform the function of structural scaffolding [133]. Because of its biocompatibility, biodegradability, and ability to provide structural support, keratin materials have found widespread use in the field of tissue regeneration [134]. Because of these features, it is feasible to manufacture a wide variety of wound dressings, such as films, hydrogels, and scaffolds, which all help to speed up the process of chronic wound healing [134].

Emulsion and coaxial electrospinning are two methods that may be used to create keratin and HA-based PCL/PEO fibers, respectively.[135] According to the findings, incorporating natural polymers into a wound dressing has the potential to improve cell survival and has applications in the field of wound dressing [135]. PVA nanofibrous scaffolds based on keratin (5% w/v) and chitosan (2% w/v) have been reported to display optimal tensile strength, swelling ratio, and porosity. These scaffolds were created using keratin. The incorporation of keratin and chitosan resulted in a significant improvement of these characteristics [136]. The same team of researchers employed the keratin and chitosan mix (50:50 by weight) in order to develop anti-inflammatory surgical sutures [136]. The scratch experiment revealed increased cell proliferation and migration in the presence of the sutures, indicating that the sutures have the potential to be employed for the purpose of promoting wound healing.

1.4.3. Casein

Casein, which originates from cow's milk, accounts for the vast majority of the protein found in milk. Because of its biocompatibility, biodegradability, and ability to self-assemble, it has attracted a growing amount of interest. Casein includes polar amino acid groups to a greater than fifty-five percent degree, which is beneficial to the creation of hydrogen bonds for the production of films [137]. On the other hand, because of this feature, it is not feasible to electrospin casein by itself. As a result, synthetic polymers such as PEG and PVA have been used in the casein-based electrospinning process in order to make it more manageable [138]. In addition, during the process of producing casein-based products, cross-linking agents such as glutaraldehyde and silane are necessary. This is because casein has a poor water stability and mechanical strength owing to the high hydrophilicity that it has [139].

Electrospun nanofibrous mats made of casein and PVA that were effective in promoting haemostatic activity through thrombin production were successfully manufactured. The inclusion of casein resulted in an increase in epithelial proliferation as well as restoration [140]. In order to speed up the healing process of wounds, casein-cellulose-chitosan scaffolds have been constructed. Because casein was present, blood coagulation ions were able to attach to exposed nucleation sites, which is necessary for maintaining hemostasis [137]. An investigation into the manufacture of an injectable hydrogel based on casein and alginate was described. Casein, alginate, nanoparticles of iron, and bacterial cellulose are the components that make up the hydrogels [138]. The fact that the hydrogels were able to absorb 4000% more water than they started with was indicative of their potential use as wound dressings. Additionally, the porosity nature of the hydrogel boosted cell viability, indicating that this particular hydrogel product may be useful in the treatment of wounds [138].

1.4.4. Hyaluronic Acid

One of the components that may be found in connective tissues is called hyaluronic acid, and it is a glycosaminoglycan. It is the primary constituent of the extracellular matrix (ECM) found in the skin, and it is also an essential component in the process by which new tissue is generated [141]. Hyaluronic acid preserves

structures that are essential for the biological function of organs, and it also has a great ability to hold water on the surface of wounds. As a result, it may protect wounded areas from drying out and hasten the healing process [142].

When used in the electrospinning process, hyaluronic acid solutions have a very high viscosity, even when used at low concentrations. This is one of the drawbacks of using these solutions. In spite of these limitations, El-Aassar *et al.* fabricated Hyaluronic acid/polygalacturonic acid nanofibrous mats embedded with silver nanoparticles [143]. An *in vivo* study demonstrated that maximum wound epithelization and collagen deposition was achieved after 14 days, indicating that its effective use for the quick healing of wound infections is possible [143]. In a separate piece of research, Eskandarinia *et al.* created polyurethane/hyaluronic acid nanofibrous mats that were infused with ethanolic extracts of propolis (EEP) at three different doses [144]. When compared with mats that contained 2% EEP, the findings demonstrated that polyurethane/hyaluronic acid nanofibrous mats with 1% EEP exhibited higher antibacterial activity and higher biocompatibility, as well as an accelerated wound healing process in the animal model. These findings supported the use of these mats for additional biomedical applications [144].

1.4.5. Silk Fibroin

The silk fibroin that is produced by a variety of insects has excellent biocompatibility, biodegradability, and mechanical qualities [145]. The use of silk fibroin as a biomedical material for the regeneration of a broad variety of tissues, including skin, vascular, cartilage, bone, tendon, and ligament, has been the subject of a significant amount of research [146]. Amino acid residues may be found in some types of silk fibroin, which are recovered from silkworm cocoons. It has been used in a number of forms (such as hydrogels, films, fibers, and sponges) to improve collagen production and re-epithelialization, which in turn enhances wound healing. These applications have been employed [147].

Electrospinning silk fibroin and nanodiamond together allowed for the fabrication of a biopolymer dressing that is capable of temperature sensing as well as wound healing [148]. In the animal investigation, researchers found that the material had a high biocompatibility and a quick rate of wound closure. It is interesting to note

that the antibacterial characteristics of this silk fibroin and nanodiamond dressing were only effective against gram-negative bacteria, whereas gram-positive bacteria were unaffected by the dressing [149]. As a result, its effectiveness as a wound dressing is up for debate given that the vast majority of gram-positive bacteria are infectious. Pacheco *et al.* stated that their research had resulted in the development of a multilayer membrane consisting of silk fibroin, chitosan, and alginate that had regulated drug release capabilities [150]. This wound dressing product benefited greatly from the use of silk fibroin due to its superior mechanical qualities. It is important to point out that the authors of this research found that the integration of the medicine into the membrane did not diminish its mechanical strength, suggesting that the dressings performed very well. This is an important finding that should be taken into consideration [150].

1.4.6. Collagen

1.4.6.1. Structure of Collagen

Collagen is the most common protein found in natural organisms including vertebrates and invertebrates. Its molecular structure is helical and consists of three polypeptide chains. This triple chain consists of α -chain subunits. Two α -chains transform into peptide chain dimer (β -peptide chain), three α -chains transform into γ -peptide chains [151].

When collagen is categorized in terms of structure, molecular organization, and non-helical domains, it can be split into fibril-forming collagens, network-forming collagens, fibril-associated collagens, linker fibrils, basement membrane collagens, transmembrane collagens. The common feature of different types of collagen structures is that they all contain carboxyl and amino ends [13].

1.4.6.2. Type of Collagen

There are currently 28 different varieties of collagen, all of which have a triple helix structure. There are differences between species in the size, shape, and length of the non-helical sections. The 5 most common types; collagen type I, II, III, VI and V

[152]. Collagen is classified into different types based on the specific composition and organization of its protein chains [152].

- **Type I:** Type I collagen chains are in a triple helical structure called heterotrimer, which consist of two same $\alpha 1(I)$ and one $\alpha 2(I)$ chain. These heterotrimeric fibers are generally included in composites consisting of type III and type V collagen [153]. Over 90% of bones are made of type I collagen, which is also the most prevalent kind of collagen. It can be present in tendons, skin, cornea, and connective tissue, among other places. Type I collagen is present in structures for instance skin, bone, organ, tendon and connective tissue, especially due to its high tensile strength, load-bearing properties and torsional stiffness [153].
- **Type II:** Type II collagen has a homotrimeric molecular structure, much like type I collagen does. Both collagen types are similar in terms of molecular size and mechanical properties. In comparison to type I collagen, type II collagen contains more hydroxylysine, glucosyl, and galactosyl residues [154]. Approximately 80% of the structure of the body's cartilage is made up of type II collagen. However, it is also found in various structures such as corneal epithelium and embryo epithelial mesenchyma [154].
- **Type III:** Type III collagen has a homotrimetric molecular structure consisting of three $\alpha 1$ chains and is usually found with type I collagen. These two different types of collagen provide the formation of mixed fibrils. It is found in the dermis, spleen, muscles and blood vessels due to the fact that the fibrils formed with type I collagen add elasticity to the body [154].
- **Type V and Type XI Collagen:** Type V and type XI collagens contain three different types of α -chains and present heterotrimer structures. It is the degree of glycosylation and hydroxylation that distinguishes type II collagen from type XI collagen, which are both encoded by the same gene. The forms of collagen that form fibrils include type V and XI collagens. They specifically combine with type I, type III, and type V collagen to generate heterogeneous fibrils. It is found in the structure of the cornea, liver, muscles, hair and placenta [154].

1.4.6.3. Sources of Collagen

Although alternatives such as human placenta and human skin-derived collagen type I are available, the most common types of collagen type I are obtained from rat tail tendon, pig, and bovine skin [155]. Human placenta and human skin-derived collagen type I are more expensive. Collagen type III has typically been extracted from cow placenta, human placenta, human skin, chicken skin, and rat skin; however, other sources, such as marine-derived sources and recombinant sources, have also been used [156].

1.4.6.3.1. Bovine Sources

Although there are other tissue sources available, research only uses collagen type III obtained from bovine skin since it is the only source from which it can be extracted [157]. As was previously indicated, variations in isolation procedures and source tissue often result in variability from one batch to the next, as well as the possibility of infectious disease contamination [158]. Even if there was no longer any danger of pathogenic contamination, the problem of inconsistency among different producers would still be a problem [158]. In addition, the distribution of proteoglycans and post-translational modifications also change with age. These changes have the potential to alter the thermal stability of tropocollagen as well as its fibrous self-assembly [159]. In general, bovine collagen is well-tolerated in vivo, and it does not trigger an immunological response in the majority of persons, with the exception of those who have a substantial allergy to collagen [159].

1.4.6.3.2. Rat Tail Tendon Sources

Given the vast quantity of published information on its isolation and characterization, rat-tail tendon, also known as rat tail tendon, is one of the sources of collagen type I that is used most often by researchers (as opposed to being utilized in industrial settings) [160]. Rat tail tendon has a collagen type I content of 90–95% by weight; the high collagen content assures considerable yields following separation [160]. Rat tail tendon collagen type I is used rather often in the field of research;

however, it is not utilized in clinical goods since there is a lack of availability of rat tail tendon collagen type I that is of a medical quality [161].

1.4.6.3.3. Marine Collagen Sources

In recent years, marine species have been the favored source of collagen due to their abundance of diverse organisms and high-quality collagen. It has the advantages of rapid bloodstream circulation, offering high absorption in the body and having a low molecular weight. Because they are biocompatible and biodegradable, they outperform other collagen sources in tissue engineering applications [162]. Marine creatures are metabolically compatible and do not contain animal pathogens, thus offering a great advantage over other sources. Collagen obtained from land animals carries many infectious diseases, which limits its industrial use. Infections in people that can be fatal can result from these illnesses. Another factor that restricts the use of animal products is the existence of religious prohibitions on their use [163].

The high rate of daily fish depletion and the abundance of by-products such as head, skin, scales, fins and internal organs as a result of this consumption create a large amount of waste called waste. This amount is thought to be more than 20 million tons [164].

Considering all these reasons, the use of marine creatures for collagen production is undeniably promising [165].

Marine collagen sources are divided into vertebrates and invertebrates. Invertebrates include shrimp, squid, octopus, sea urchin, and sponges. The vertebrate group consists of fish and marine mammals. Collagen, which can be obtained from fish by-products, offers a potential source for recovery and sustainability. These by-products include fins, skins and scales. Collagen can be obtained after these products go through various bioprocessing processes [166].

Mammals commonly used for collagen production; whales, otters, and seals. The results of the researches have revealed that collagen derived from pigs, cattle, and marine organisms has similar amino acid compositions. The features that make the difference are that the collagen obtained from fish contains less proline and hydroxyproline than the collagen obtained from mammals. This difference causes a

decrease in the stability and cross-linking compatibility of collagen obtained from fish [167].

The most frequently extracted kind of collagen from marine species is type I, however type II collagen can also be found in things like fish cartilage and Strip Jellyfish (*Chrysaora*) [168]. In addition, there are studies in the literature that obtained type IV collagen from sea sponges such as *hondrosia reniformis*.

We used fish scale in this thesis study for obtaining of collagen because fish collagens provide a number of advantages, including a lower risk of disease transmission and an abundance of collagen-containing tissues.

1.4.6.3.4. Fish

Fish components like scales, fins, skin, and bones can be used to make collagen (Figure 1.4) [169]. Although it varies according to the fish species, age, season, and where it grows and develops, fish skin is predominantly composed of type I collagen. This variation is because different tissues have different needs and may adapt in a variety of ways in response to changing physiological and environmental situations [170]. Fish collagen is a good source of collagen since it doesn't irritate people or animals and has a high ability to hold water, making it ideal for cutaneous applications [171].

Fish by-products are rich in protein content. For this reason, these products, which are called waste, can be used as plant fertilizer, protein powder or animal feed [172].

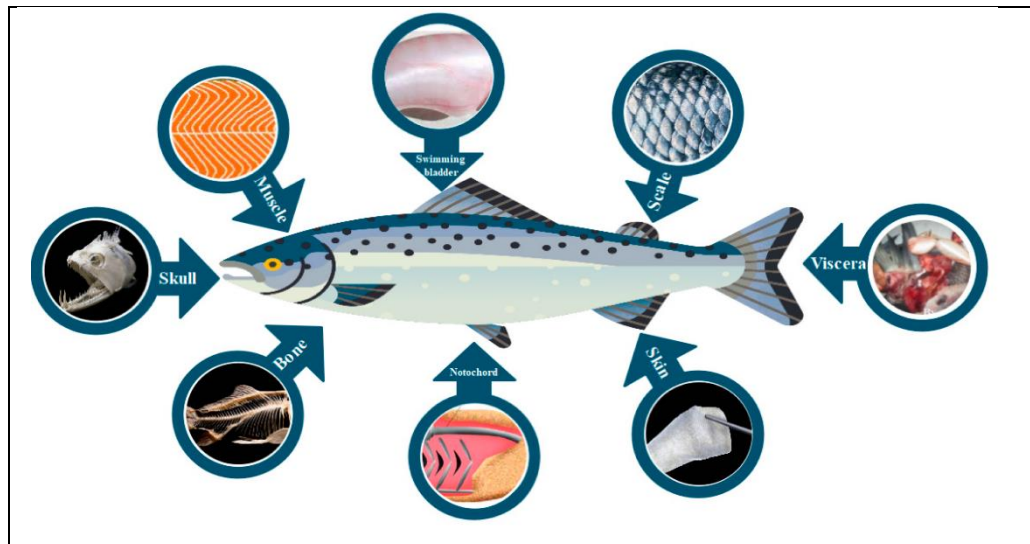


Figure 1.4: Fish by-products that might be used as possible sources of collagen extraction [172].

- Fish Skin:** Even though Type I collagen fluctuates depending on things like fish species' ages and seasonal conditions, it makes up about 70% of fish skin [173]. The collagen produced by processing fish skin using a number of processes is appropriate for use in dermal applications because it has advantages including being biocompatible, not irritating the skin, and having a high water holding capacity (approximately 6% of its weight when treated with 63% moisture for 24 hours) [171]. Blanco *et al.* used two species of Chondrichthyes and two species of teleost in their research and gathered collagen from fish skins [173]. While collagen from cod fish was denatured at 16 °C, collagen from two species of Chondrichthyes and two species of teleost revealed denaturation temperatures in the range of 23-33 °C. This temperature difference indicates the habitat difference of the species [174]. In comparison, the temperature required to extract collagen from the skin of catfish, pomfret, tilapia, and mackerel is lower (less than 13 °C), and the process takes more time (74 hours). It demonstrates that the typical collagen output from these species is around 2.27% [175]. In addition to this study, Wijaya *et al.* investigated the collagen's allergenic properties using Parang-Parang fish skin. [176]. Acetic acid was used to hydrolyze collagen after it had been extracted using NaOH. It was discovered that the yield of protein content other than collagen was 1,915%.

Govindharaj *et al.* obtained collagen from eel skin for use in 3D printing application. Collagen had a final yield of approximately 4.2% [177]. In a comparable

result, the yield of the collagen recovered from eel fish was reported to be 4.7% [178]. Ahmed *et al.* used bacterial collagenolytic proteases (CP) produced by using *Bacillus cereus* FRCY9-2 and *Bacillus cereus* FORC005 to obtain collagen from fish skin in their research [179]. The overall yield of collagen from the bacteria treatments and when combined with acid-soluble collagen was 188 and 177 g/kg, respectively, which was greater than the yield attained through acid extraction alone (134.5 g/kg). In contrast to these techniques, collagen from the skin of *Atlantic cod* (*Gadus morhua*) was produced using CO₂-acidified water in another study [180]. Collagen that had been extracted using acidified water had 151/1000 residues of proline-like amino acids overall, with a 13.8% (w/w) extraction yield [180].

Afzali *et al.* generated collagen by employing the skin of *Lutjanus* sp in their study [181]. Their goal was to establish an ideal combination of fish skin collagen, sodium alginate, and hyaluronic acid loaded with bovine serum albumin as a model protein manufactured as lyophilized scaffolds. Collagen was recovered from the fish skin by him and his coworkers using a technique known as semi-bionic extraction (SBE). They were successful in obtaining composite gels made of collagen, sodium alginate, and hyaluronic acid. In vitro blood study utilizing human whole blood indicated that the BSA-loaded SA*:FCOL:HA scaffolds lowered the blood clotting index (BCI) by up to 20% compared to a commercially available sponge for chronic wounds. This was determined by comparing the two products using human whole blood. The presence of these characteristics demonstrates that SA*:FCOL:HA scaffolds have the potential to serve as multifunctional wound dressings [181].

In the research conducted by Zhou *et al.*, fish collagen was extracted from the skin of tilapia [182]. Electrospinning was used to produce nanofibers that are biomimetic and are composed of collagen and BG. They looked at their structures, as well as their tensile strengths, antibacterial activities, and the biological impacts that they had on human keratinocytes, human dermal fibroblasts, and human vascular endothelial cells. The findings demonstrated that in comparison to pure fish collagen nanofibers, the tensile strength of the Col/BG nanofibers rose to 21.87±0.21 Mpa, and they had a considerable degree of antibacterial activity against *Staphylococcus aureus*. In addition to this, it was discovered that the Col/BG nanofibers encouraged human keratinocytes to adhere to one another, proliferate, and migrate [182].

- **Fish Bone:** Type I collagen, which is composed of two $\alpha 1$ and one $\alpha 2$ chain combinations, is present in fish bone. [183]. The research in the literature, which sought to get collagen from the bones of tilapia (*Oreochromis mossambicus*), revealed a denaturation temperature of 32.5 °C [184]. Collagen from the bones of catfish, mackerel, pomfret, and tilapia species has a higher temperature value (16.6-19.03 °C) and requires less time to extract (73.16 hours) than collagen extracted from the flakes and skins of these species. However, its extraction efficiency is lower (0.64%) [185].

Ramli *et al.* obtained collagen by using *Lutjanus sp* bone in their research and they claimed that this collagen can be employed as an anticancer drug [186]. In this research, the collagen production yield was found to be 4.535 [187]. He and his colleagues removed calcium and collagen from fish bones, which are referred to as garbage, using semi-bionic extraction (SBE) technology and pulsed electric field (PEF) treatments. The SBE method mimics the absorption and digestion procedure of the human gastrointestinal system by using a range of pH values as well as acid and alkali extraction procedures. In this research, 22.79 kV/cm PEF was used and they obtained 3.87 mg/mL collagen. Calcium and collagen were obtained from fish bone by combining SBE and PEF techniques [186].

In other research conducted by Ramli *et al.* synthesized collagen from fish bone using 1% pepsin and a PEF strength of 20 kV/cm. A collagen output of 16.13 mg/mL was found in the evaluation carried out as a result of this study. According to these findings, the fundamental desalination procedure should involve employing ethylenediamine tetraacetic acid (EDTA) and hydrochloric acid (HCl) to remove calcium and high hydroxyapatite concentrations. It should be noted that the HCl employed in this procedure may weaken collagen's structure [184].

In Wang *et al.* study, chitosan and chum salmon (*Oncorhynchus keta*) skin, bone, and scales were used to generate a collagen composite gel [188]. Following the characterization of this composite gel, its biocompatibility was investigated, and an inflammatory response was reported. The specimens were collected in order to conduct a histological examination in addition to an ELISA-based tumor necrosis factor-alpha (TNF-) analysis. When the ratio of salmon atelocollagen to chitosan was either 10:90 or 20:80, the inflammatory cell infiltration as well as the production of TNF- were repeatedly regulated at low levels. After implantation, initially the salmon atelocollagen gel, and then the chitosan in the composite gel, were progressively

absorbed, and this was followed by the creation of soft tissue. This process took around 2 weeks [188].

- **Fish Cartilage:** Fish cartilage has a high amount of collagen, the majority of which is type II collagen, while Hoki (*Macruronus novaezelandiae*) is known to contain a small amount of type IX and XI collagen [171]. Fish species such as *Dasyatis akjei*, *Raja porosa* and *Sphyrna lewini*, on the other hand, have type I collagen in their cartilages. There is type II collagen present, despite the fact that type I collagen is known to make up the majority of the cartilage in the *Amur sturgeon* (*Acipenser schrenckii*) [187].

Comparable to mammalian cartilage in terms of amino acid content and chain arrangement is the collagen produced from Hoki cartilage. For the treatment of disorders related to cartilage, this kind of collagen may have application as biomaterials [187]. Due to the high quantities of antioxidants found in silvertip shark collagen, this species is chosen for use in biomedical applications. Jeevithan *et al.* investigated the production of collagen from Silvertip shark (*Carcharhinus albimarginatus*) and the antioxidant and physicochemical features of this collagen [189]. The collagen types obtained are as follows; type II gelatin, acid-soluble type II collagen (ASC), and pepsin-soluble collagen (PSC).

Luo *et al.* obtained ASC with $27.13\% \pm 1.15\%$ yield and PSC with $14.69 \pm 0.85\%$ yield by using cartilages of *Siberian sturgeon* (*Acipenser baerii*) in their research [190]. This type of collagen has the potential to be an alternative to type II collagen obtained from mammals. Luo *et al.* obtained ASC with $27.13\% \pm 1.15\%$ yield and PSC with $14.69 \pm 0.85\%$ yield by using cartilages of these fish cartilage in their research [190]. The possibility exists that this kind of collagen could replace type II collagen derived from mammals.

Collagen from cartilage demonstrates a lower temperature of denaturation than the $26.3\text{ }^{\circ}\text{C}$ to $35.9\text{ }^{\circ}\text{C}$ denaturation temperature typical of bovine collagen [191]. For example, collagen from trout (*Oncorhynchus keta*) is denatured at $19\text{ }^{\circ}\text{C}$, while collagen from shark is denatured at $30\text{ }^{\circ}\text{C}$ [192]. This discrepancy between temperatures limits its medical applications on humans.

The acid solubilized collagen (ASC) and pepsin solubilized collagen (PSC) were isolated from the by-products (bone) of a cartilaginous fish (*Mustelus mustelus*) in the investigation that was conducted by Slimane *et al.* [83]. Chitosan and collagen that had

been solubilized with pepsin were used to make composite films. When compared to chitosan film, this kind of film had a lower tensile strength but a greater elongation at break; when compared to collagen film, this type of film exhibited a lower water solubility and a lighter weight. In the same vein, an examination of the film composite using FTIR spectroscopy demonstrated the presence of a collagen-chitosan interaction that led to an alteration of the secondary structure of collagen. The collagen-chitosan biofilm shown potential UV barrier qualities as well as antioxidant activity; hence, it has the potential to be used as a green bioactive film for the preservation of nutraceutical items [83].

- **Fish Scale:** Fish scales can cause serious waste generation in the fish processing industry due to high volume, lack of utilization, difficult to dispose, environmental concerns, odor and pest attraction, missed opportunities. Type I collagen can be obtained from these wastes, thus the wastes are evaluated [193]. In a research aimed at obtaining collagen using Tilapia (*Oreochromis* sp.) scales, it was observed that this collagen has high amino acid content, Van der Waals interactions and high intra/inter-chain and ionic bonds therefore has a high denaturation temperature [189].

On the other hand, collagen was found to be produced from fresh carp (*Cyprinus carpio*) scales at a low denaturation temperature (32 °C) [194]. The yield of collagen from the skin of pomfret, catfish, mackerel, and tilapia species is 4.3%; however, the yield of collagen from their scales is only 0.13%. However, obtaining collagen from scales requires a longer extraction time of approximately 77 hours and a higher extraction temperature (16.6–19.03 °C) [185]. Fish scales are a suitable source for collagen production in terms of their water holding (15%) and water absorption (13.3%) capacities required for medical and biomedical applications [189].

1.5. Applications of Fish-Derived Collagen on Wound Healing

Wound healing includes a difficult process due to the fact that the healing process of the wound starts from coagulation and ends in re-epithelialization consists of many phases. The use of traditional drugs in wounds, such as burn reactions, may cause the person to develop drug resistance. For this reason, natural origin treatment

methods should be investigated. With the prolongation of the healing process, various complications and pain may develop. Collagen is a good contender for biomedical applications due to its biocompatibility, capacity to penetrate more lipid-free interfaces, and biodegradability. Thanks to the high strength and stable structure of collagen, its fibers can be self-organized and cross-linked, making it important for biomedical applications [195].

There are many studies in the literature examining the usage of collagen hydrogels in wound dressing. The reason is that it provides a three-dimensional structure that resembles the moisture in the cell structure of the skin and provides the moist environment that wounds need [196].

Collagen can be seen as an great option for wound healing by the reason of its non-antigenicity, impermeability to bacterial contamination and air permeability. Thanks to its flexible and elastic structure, it shortens the recovery time and reduces pain. It fosters the growth of cells and tissues while encouraging the synthesis of cell tissue matrix. It stimulates the formation of fibroblasts and keratinocytes, which accelerates the healing process in the wound area. Fish collagen has wound healing qualities that are antibacterial, anti-inflammatory, and immunological modulatory [197].

Elbially *et al.* investigated the impact on cutaneous wound healing by obtaining type I collagen from the skin of Nile tilapia (*Oreochromis niloticus L.*) in their research on rats [196]. It was shown that the obtained collagen had a stimulating impact on wound healing since it promoted keratinocyte proliferation, led to fibroblast differentiation, and increased the formation of ECM. In another research conducted by Shalaby *et al.*, collagen was obtained from mullet and tilapia fish and its effect on wound healing was investigated [195]. As a result of the research, it was seen that collagen increased the cell adhesion capacity, thus promoting wound closure and accelerating its healing. The presence of hydroxyproline, an element of collagen, can be used to monitor the accumulation of collagen in a certain area and thus wound healing. Collagen promotes in the creation of granulation tissue, re-epithelialization of the wound, angiogenesis, and platelet aggregation, all of which contribute to the promotion of wound healing.

1.6. Sodium Alginate

Brown seaweed is the source of the biopolymer known as alginate, which has a naturally occurring anionic structure and is produced as a biopolymer. It is easily accessible, is biocompatible, and does not cause any harm [198]. The moist environment and lower risk of bacterial infections that are hallmarks of alginate-based wound dressings are two of the most significant contributors to the speedy recovery of wounds [198].

Some researchers have created and reported on the possible use of wound dressing materials that are films based on sodium alginate [199]. They protect the wound from bacterial infections, speed up the process of wound healing, increase permeability to water vapor, carbon dioxide, and oxygen, and alginate's mechanical qualities may be increased by mixing it with other polymers.

Liakos *et al.* produced films made of sodium alginate that had essential oils infused into them [200]. Glycerol was utilized as a plasticizer, and various essential oils such as chamomile blue, cinnamon, lavender, tea tree, peppermint, eucalyptus, lemon grass, and lemon oils were put into the films [200]. Igepal surfactant was used so that we could improve the dispersion of the oil throughout the matrix of the film. Because of the presence of the surfactant, the miscibility of the essential oil and the matrix was significantly improved. The films demonstrated a high level of antibacterial activity. The films that included tea tree, cinnamon, lemon grass, and peppermint were successful in inhibiting *E. coli* and *C. albicans* at all of the concentrations that were tested. Only *Candida albicans* was susceptible to the killing effects of the film that included elicriso oil [200]. The kind of essential oil that was placed onto the films had an impact on the selectivity of the films' antibacterial activity, which was measured in micrograms per square centimeter.

Pereira *et al.* produced hydrogel films were made by using alginate and aloe vera gel in several quantities, including 75:25, 85:15, and 95:5 by volume/volume respectively [201]. Their swelling increased with an increase in the quantity of aloe vera that was included in the films, and their mechanical qualities were acceptable for applications that involved the skin. The films had a surface that was very smooth, they were quite malleable, and their thicknesses ranged from 66.14 to 69.00 micrometers. Tensile strength and elongation values at the point of breakage were found to vary

from 21.44 to 40.44 MPa and 5.94 to 13.27%, respectively, in the films that were not crosslinked. Because of the addition of glycerol, there was a significant reduction in the stiffness of the material. This was shown by a reduction in the tensile strength in the range of (40.44–28.66 MPa) and an increase in the elongation at break in the range of (5.94–13.27%) [201].

1.7. Applications of Sodium Alginate on Wound Healing

Due to its simplicity of gelation, biocompatibility, bioresorption, sodium alginate has a variety of uses in wound healing. It is frequently utilized as a hydrogel for a variety of biological applications, such as tissue engineering, medication delivery, and wound healing [202].

Because they may gel when they come into touch with moisture, sodium alginate hydrogels have been used as dressings in the field of wound healing [203]. These hydrogels have a great capacity for absorbing moisture, which aids in preserving a moist environment for comfortable wound healing. The hydrogel matrix of sodium alginate also enhances the water vapor transmission capacity, allowing for better wound healing [16]. It has been discovered that sodium alginate hydrogels exhibit outstanding biological affinity and adhesive characteristics [204]. Their limited clinical applications are due to the fact that their adhesive strength declines under physiological status [205]. To address this issue, recent trends have focused on developing hybrid or composite hydrogels using sodium alginate to enhance the mechanical features of wound dressings [204]. Additionally, it has been noted that sodium alginate hydrogels possess antibacterial qualities, making them appropriate for the treatment of wounds [206]. Alginate hydrogels containing Aloe vera and essential oils have been used in clinical wound care due to their antimicrobial and anti-inflammatory capacities [205]. These hydrogels can serve as disposable wound dressings with antimicrobial properties [206].

Researches have also investigated the wound healing effects of sodium alginate in combination with other bioactive agents. For instance, a study demonstrate that the topical application of lupeol, combined with sodium alginate gel, enhanced considerable wound healing activity [207]. Sodium alginate has also been used as a matrix material in hybrid formulations with polyvinyl alcohol and bioactive agents like

GSNO (S-nitrosoglutathione) for wound dressing applications [208]. Sodium alginate has been coupled with bioactive substances to treat diabetic wounds in addition to being used as a material for wound dressings. For diabetic wounds, for instance, sodium alginate-gelatin wafers containing diosmin nanocrystals have been created [209]. Additionally, silymarin-loaded sodium alginate-based wafers have been demonstrated to outperform the high glucose-induced reduction in endothelial cell migration, highlighting their potential for managing diabetic wounds [209].

Sodium alginate films have been extensively researched and applied in wound healing due to their favorable properties and ability to form hydrophilic gels. These films, which include films, hydrogels, wafers, foams, and nanofibers, can be customized to fit various types of wound dressings [16]. One of the key advantages of sodium alginate films is their ability to absorb excess wound fluid and pursue a moist environment, which is vital for optimal healing process [16]. These films can create a moist microenvironment that promotes cell migration, angiogenesis, and collagen synthesis [91]. The maintenance of a moist environment also helps in preventing wound dehydration and scab formation, which can impede the healing process [16]

Alginate films can also bind pathologic elements that are harmful to wound healing, such as proteases and proinflammatory cytokines, helping to foster an environment that is conducive to healing [210]. The therapeutic efficacy of sodium alginate films can be further enhanced by incorporating other bioactive agents. For example, alginate films loaded with essential oils have demonstrated remarkable antimicrobial and antifungal properties, making them suitable for wound dressings [211]. Furthermore, sodium alginate films have been found to be biocompatible and non-toxic, making them safe for use in wound healing applications [16]. It has been demonstrated that they encourage cell adhesion, proliferation, and migration that is the processes crucial for the healing of wounds [89]. Alginate films can also provide thermal isolation, relieve pain, and be easy to use [89].

2. LITERATURE REVIEW

George *et al.* created films that included gelatin as well as sodium alginate using a procedure called solvent casting [212]. After that, they loaded the films with silver sulfadiazine. The films were capable of entrapping between 93% and 97% of the silver sulfadiazine and were biodegradable. The picture had a release profile of silver that was maintained over the course of seven days. The films had a folding capability that ranged from 337 ± 0.96 to 459 ± 0.69 , which is indicative of their capacity to endure the mechanical pressure and excellent flexibility. It was found that the films had an encapsulation percentage ranging from 90.23% to 97.75% of the medication, making them suitable for use as wound dressing [212].

In the research conducted by Feng *et al.*, an improved method for full-thickness wound healing was devised using a hybrid hydrogel that included aminated collagen, oxidized sodium alginate and antimicrobial peptides (polymyxin B sulfate and bacitracin) [213]. The aminated collagen with low immunogenicity and good biocompatibility was created using marine fish scales, which are sustainable, eco-friendly, and inexpensive. The aminated collagen also has a high degree of immunogenicity. A Schiff base reaction was used, however no catalysts were necessary, and extra steps were not required in order to produce the cross-linked hydrogel. As was to be predicted, the hybrid hydrogel that was shown has the ability to successfully combat *E. coli* and *S. aureus*, in addition to promoting cell development and angiogenesis in vitro. The findings of the in vivo assessment indicated that the hybrid hydrogel dressing has the ability to improve full-thickness wound healing by fostering the creation of granulation tissue and the deposition of collagen, as well as accelerating angiogenesis and epithelial regeneration. In addition, a rat model has shown that the hydrogel may accelerate the process of reepithelialization, collagen deposition, and angiogenesis, all of which are necessary for full-thickness wound healing. The results of their research showed that the hybrid hydrogel might potentially have useful uses in the area of wound healing [213].

Li *et al.* produced strontium loaded silk fibroin-sodium alginate films for the purpose of wound dressing [214]. The amount of strontium that was placed onto the films varied from 1 mg/mL all the way up to 30 mg/mL. The amount of strontium placed into the films caused the water absorption rate to drop, which was inversely

proportional to the amount of strontium present. The water absorption rate of films that do not include strontium was found to be 900%, while the water absorption rate of films containing either 1 mg/mL or 30 mg/mL of strontium was found to be either 800% or 100%, respectively. According to these observations, following the incorporation of strontium, the films acquire a greater degree of hydrophobicity. The water vapor transfer rate of the films was in the range of 2,000 to 2,700 g/m² per day, which is enough to prevent excess exudates at the wound site, which may lead to bacterial infections and slow the healing process [214].

In the research conducted by Liu *et al.*, a collagen-chitosan composite film called CC-G-E film was modified using grapheme oxide (GO) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) [215]. This film was then loaded with basic fibroblast growth factor (bFGF) in an effort to build an effective wound healing device. They describe in this research a unique drug delivery system that inhibits the initial burst release of pharmaceuticals as well as the loss of bioactivity of medications when used in *in vitro* and *in vivo* settings respectively. When the dosage of GO was between 0.03% and 0.07%, the findings revealed that the CC-G-E film had enhanced thermal stability, a greater rate of crosslinking, and increased mechanical characteristics. These results were shown when the dosage of GO was between 0.03% and 0.07%. The *in vitro* release of bFGF from CC-G-E film was shown to persist for more than 28 days. In addition, the CC-G-E films showed good *in vitro* biocompatibility after being cultured with L929 fibroblasts, exhibiting great cell attachment and proliferation as a result of the culture. The capacity of Sprague Dawley rats to mend full-thickness skin wounds was evaluated by implanting CC-G-E films into their skin and observing the results. In comparison to the control with no treatment, the CC-G-E film was shown to hasten the process of wound healing in the study's findings [215].

Rezvanian *et al.* revealed a composite film made of sodium alginate combined with pectin or gelatin [216]. According to the results of the pre-formulation investigations, the combination of sodium alginate and pectin produced outstanding mechanical qualities. It was discovered that an alginate/pectin composite film filled with simvastatin might be a suitable and effective wound dressing. The films themselves were non-toxic. When compared to the apparent viscosity of other gels, the sodium alginate-gelatin film loaded with simvastatin had a value of just 250 s⁻¹. This represented a considerable reduction in viscosity. The ratio of the polymers in the film

had an effect on the pace at which the medication was released from the film. The medication was released at a rate of 0.826 ± 0.069 mg/cm² from sodium alginate-pectin film loaded with simvastatin, 0.929 ± 0.101 mg/cm² from sodium alginate loaded with simvastatin, and 1.736 ± 0.134 mg/cm² from sodium alginate-gelatin loaded with simvastatin, respectively. Evaluation of cytotoxicity was out in vitro on normal human dermal fibroblast cells demonstrated a cell viability of 83.20 percent in comparison to cells that had not been treated [216].

Dantas *et al.* described the use of sodium alginate-chitosan based films for the treatment of burns in conjunction with laser therapy [217]. In vivo investigations conducted on male rats utilizing a combination of laser treatment and the films boosted the quick replacement of type III collagen with type I collagen, blood vessel creation, epithelization, collagenization, and encouraged excellent arrangement of the newly generated collagen fibers. The combination enhanced the biostimulatory effect of low-level laser treatment, which led to a considerable improvement in the rate at which the burns healed [217]. This improvement was due to the anti-inflammatory properties of chitosan and alginate.

Sharma *et al.* produced films containing silver nanoparticles, sodium alginate, and chitosan by with varying ratios [218]. In comparison to other films, the one with a sodium alginate-chitosan films ratio of one to one displayed a larger percentage of water absorption, improved mechanical qualities, was simple to handle, and had a smoother surface. It had a thickness of 5.562 ± 0.43 millimeters, an elongation value at break of $23.80 \pm 2.53\%$, a tensile strength of 16.55 ± 3.62 megapascals, and a Young Modulus of $520\ 88.82$ megapascals, which was high in comparison to other ratios. In addition, the films were successful in inhibiting Gram-positive bacteria, which further demonstrated their potential use in wound dressing [218].

3. MATERIALS and METHOD

In this section, the materials required for collagen-sodium alginate composite film structures produced within the scope of this thesis, the experimental stages and characterization methods are included. In the first stage of the experiment, type I collagen is extracted from sea bass fish (*Dicentrarchus labrax*) scales. The second stage is the preparation of collagen and sodium alginate composite film using solvent casting technique. The last step involves the incorporation of characterization procedures in order to conduct thermal, chemical, morphological, and elemental analyses of the collagen and films that have been produced.

3.1. Materials and Devices

The table below lists the precursor materials that were used in the experimental stages that was carried out.

Table 3.1: Materials used in whole experimental stages of this thesis research.

Name	Brand	City/Country
<i>Dicentrarchus labrax</i> scale	Sürsan	Muğla/Turkey
Sodium chloride	Isolab	Wertheim/Germany
Acetic acid (Glacial)	Tekkim	Bursa/Turkey
Hydrochloric acid	Honeywell	Seelze/Germany
Pepsin (From porcine gastric mucosa)	Millipore Corporation	Darmstadt/Germany
Sterile gauze compresses	Kuteks	Aydın/Turkey
Glycerol	Sigma-Aldrich	Schnelldorf/Germany
Glutaraldehyde	Sigma-Aldrich	Schnelldorf/Germany
Visking dialysis tube	Serva	Heidelberg/Germany
Sodium alginate	Sigma-Aldrich	Schnelldorf/Germany
Sterile gauze compresses	Kuteks	Aydın/Turkey

3.2. Methods

3.2.1. Preparation and Demineralization of Fish Scales for Extraction

After the scales were obtained from Sürsan (Muğla/Turkey), they were stored at -20°C until used after washing with distilled water. As seen in the Figure 3.1, in order to get rid of non-collagenous proteins that may be on the surface of the scales, they were first washed 2 times in 10% NaCl solution, then mixed for 24 hours (Scale:solution ratio: 1:10). The scales were demineralized so that they could be used in the collagen extraction process without any further preparation. In this context, the scales were mixed with 0.4 mol/l of HCl (Scale: solution ratio: 1:15) for 90 minutes in order to demineralize them. After that, the scales were washed three times with distilled water in order to prepare them for collagen extraction.

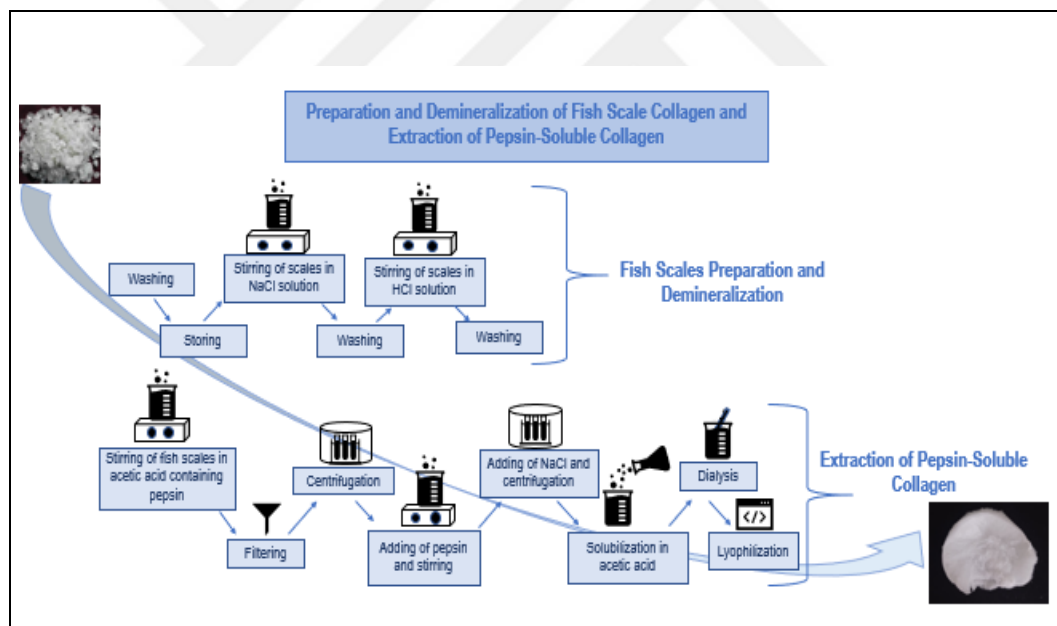


Figure 3.1: Schematic representation of preparation and demineralization of fish scale collagen and extraction of pepsin-soluble collagen process.

3.2.2. Extraction of Pepsin-Soluble Collagen from Fish Scales

The extraction process of collagen was carried out at $+4^{\circ}\text{C}$. The scales were mixed for 48 hours in 0.5 M acetic acid solution containing pepsin (1 wt.% of the

demineralized scales) (Figure 3.1). Then, the solution was filtered with the help of gauze in order to remove the residues that may be present in the solution. The filtered solution was centrifuged at $9000 \times g$ for 60 min. Pepsin (1 wt.% of the estimated collagen) was added to the supernatant and mixed for 48 hours with magnetic stirrer. Then, the aqueous solution with 1 M NaCl added was mixed in 0.5 M acetic acid for purification after centrifugation at $9000 \times g$ for 60 min. The solution obtained at the end was put into a dialysis tube and dialyzed in distilled water containing 0.1 M acetic acid for 3 days. These membranes were used in this thesis work as they are semi-permeable membranes are designed to enable tiny molecules to flow through, such as salts, solvents, and small peptides, while preventing bigger molecules, such as collagen, from passing through. It was then lyophilized at -20°C for 24 hours.

3.2.3. Preparation of Fish Scale-Derived Collagen and Sodium Alginate Composite Film

A solution was prepared by adding collagen (10%, w/v) to distilled water and stirred for 30 minutes at 65°C on a magnetic stirrer (Figure 3.2). Then, different ratios of sodium alginate (Collagen: sodium alginate: 10:0, 10:3 and 0:2) were added to the aqueous collagen solution and mixed for another 30 minutes at 65°C . Film solution was prepared by adding glycerol and glutaraldehyde (0.5%, v/v) at a rate of 20% by dry weight to the mixture. The prepared solution was poured into petri dishes and left to dry for three days at room temperature (25°C).

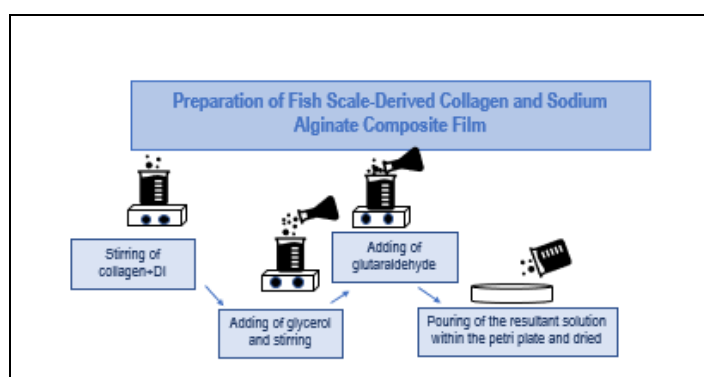


Figure 3.2: Schematic representation of preparation of fish scale-derived collagen film, sodium alginate film, fish scale-derived collagen and sodium alginate composite film process.

3.2.4. Characterization of Fish Scale-Derived Collagen Film, Sodium Alginate Film and Fish Scale-Derived Collagen and Sodium Alginate Composite Film

FTIR analysis was performed to investigate determining the chemical composition, interactions, and molecular structure of collagen, collagen film, sodium alginate film, and sodium alginate composite films. SEM-EDS analysis was performed to investigate particle and fiber distribution, morphology examination, elemental analysis, microstructural analysis of collagen, collagen film, sodium alginate film, and sodium alginate composite films at the micro and nanoscale. In order to examine thermal behavior analysis and discover phase changes in collagen, collagen film, sodium alginate film, and sodium alginate composite films, DSC analysis was carried out. TGA analysis was performed to investigate thermal stability and decomposition behavior of collagen, collagen film, sodium alginate film, and sodium alginate composite films. In order to examine the crystallographic structure and crystalline phases of collagen, collagen film, sodium alginate film, and sodium alginate composite film, XRD analysis was carried out.

3.2.4.1. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

FTIR analysis was performed to examine the interactions of collagen and sodium alginate composite films derived from fish scale, collagen and sodium alginate compounds with each other. Samples taken from the collagen, collagen film, sodium alginate film, and sodium alginate composite film were analyzed using infrared spectrometer (Spectrum 100, PerkinElmer, USA) and measurements made in the range of 400-4000 cm^{-1}).

3.2.4.2. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) Analysis of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

Scanning Electron Microscope (SEM) (Win10 FEI (Philips) XL30 microscope, USA) analysis was performed in order to examine the topography and morphology of the collagen, collagen film, sodium alginate film, and sodium alginate composite films. The samples taken from the collagen, collagen film, sodium alginate film, and sodium alginate composite films were coated with gold and prepared for analysis.

Energy dispersive spectroscopy (EDS) was used in this technique to assess the weight percentage of elements in the collagen, collagen film, sodium alginate film, and sodium alginate composite films. The EDS spectra of collagen, collagen film, sodium alginate film, and sodium alginate composite films were examined using EDAX Genesis software at a working distance of 15 mm and an acceleration voltage of 5 kV.

3.2.4.3. Differential Scanning Calorimetry (DSC) Analysis of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

The device (DSC 404 C Pegasus, Netzsch-Gerätebau GmbH, Germany) was used to examine the thermal properties of collagen, collagen film, sodium alginate film, and sodium alginate composite films. Before the measurement was taken, the samples were weighed and the analysis put into the aluminum pan which hold the sample during the analysis and to facilitate accurate temperature measurements. The analysis was performed at the determined measurement temperature range of -30°C to 400°C.

3.2.4.4. Thermogravimetric Analysis (TGA) Analysis of of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

It was used to examine the composition, thermal behavior, stability, moist content of the sample by measuring the weight change of the collagen, collagen film, sodium alginate film, and sodium alginate composite films depending on temperature or time. The samples were placed on the pans to be measured and put into the device (TGA Linseis STA 1600 HP, Germany), and the measurement was taken by heating in the temperature range of 25°C to 900°C.

3.2.4.5. X-Ray Diffraction (XRD) Analysis of of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

The purpose of the usage XRD analysis examine crystallinity assessment, phase identification, crystalline behavior of collagen, collagen film, sodium alginate film, and sodium alginate composite films. It was aimed to examine the atomic and molecular order in X-rays interact with the crystal lattice in the structure of the film and analysis is provided by analyzing these interaction forms. Cu Ka1 radiation was used for measurement and recorded with a Bruker D-8 Advance XRD instrument. A Rigaku D-Max 2200 PCI diffraction meter was used to captured the diffraction patterns over 2 within 5-80°.

4. RESULTS & DISCUSSION

Chemical composition, interactions and molecular structure of collagen, collagen film, sodium alginate film and sodium alginate composite films were determined by FTIR analysis. With SEM-EDS analysis, particle and fiber distribution, morphological analysis, elemental analysis, microstructural analysis of collagen, collagen film, sodium alginate film and sodium alginate composite films were investigated at micro and nano scale. Thermal behaviors and phase changes in collagen, collagen film, sodium alginate film and sodium alginate composite films were investigated by DSC analysis. Thermal stability and decomposition behavior of collagen, collagen film, sodium alginate film and sodium alginate composite films were investigated by TGA analysis. Finally, the crystallographic structure and crystal phases of collagen, collagen film, sodium alginate film and sodium alginate composite film were investigated by XRD analysis.

4.1. Results of FTIR Analysis of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

The FTIR spectra of collagen from the *Dicentrarchus labrax* scales was depicted in Figure 4.1.

a)

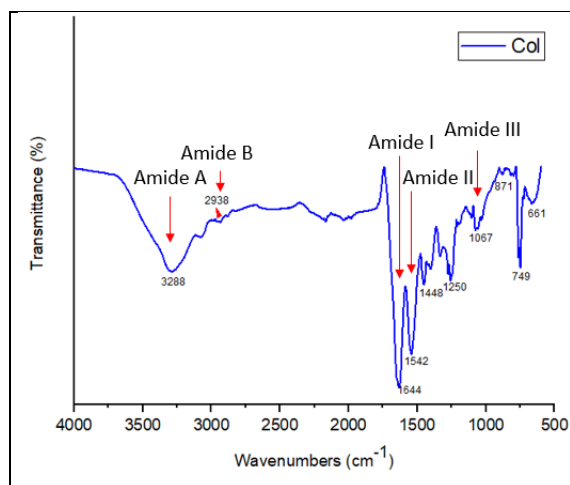


Figure 4.1: FTIR spectra of collagen from *Dicentrarchus labrax* scales.

As seen in Figure 4.1, the characteristic peaks of collagen from *Dicentrarchus labrax* scales was found at 3288 cm⁻¹, 2938 cm⁻¹, 1644 cm⁻¹, 1542 cm⁻¹, 1448 cm⁻¹, 1250 cm⁻¹, 1067 cm⁻¹, 871 cm⁻¹, 749 cm⁻¹, and 661 cm⁻¹. These values line up with the research conducted by El-Rashidy *et al.* [219]. As demonstrated, the amide A absorption band, which is connected to N-H stretching and hydrogen bonding, is found at 3288 cm⁻¹, was found to be similar with the results achieved in the study conducted by Purna Sai *et al* [220]. Additionally, locations of the amide B band were discovered at 2938 cm⁻¹, which were connected to an asymmetric stretch of CH₂, were found to be similar with the results achieved in the study conducted by Abe *et al.* [221]. The amide I band in 1644 cm⁻¹ is connected with carbonyl group vibrations, that is coupled with N-H bending vibrations. This suggests that the triple helix was formed as a result of hydrogen bonds forming between C = O groups and N-H groups, CN stretching, and CCN deformation [222].

The peak observed at 1542 cm⁻¹ was associated with the NH vibration of in-plane bending and combination of CN stretching (Amide II), which was matching with the results achieved in the study conducted by Duan *et al.* [183]. At 1448 cm⁻¹, peaks were observed in association with CH₂ bending (Amide II), which was matching with the results achieved in the study conducted by Jackson *et al.* [223]. As a result of amide linkage, the C-N stretching and N-H in-plane bending that constitute up the complicated amide III band in 1250 cm⁻¹ are both present. Additionally, it is created

by the absorption of wagging vibrations from CH₂ groups on the side chains of proline and the glycine backbone [222]. The peak observed at 1067 cm⁻¹ was associated with the C-O stretching (Amide III), which was matching with the results achieved in the study conducted by Jackson *et al.* [223]. At 871 cm⁻¹, 749 cm⁻¹ and 661 cm⁻¹ peaks were observed in association with skeletal stretching, were found to be similar with the results achieved in the study conducted by Abe *et al.* [221]. The outcomes are consistent with Pati *et al.*, Duan *et al.*, and Muyonga *et al.* [224, 225].

The FTIR spectra of collagen film, sodium alginate film, collagen and sodium alginate composite films were depicted in Figure 4.2.

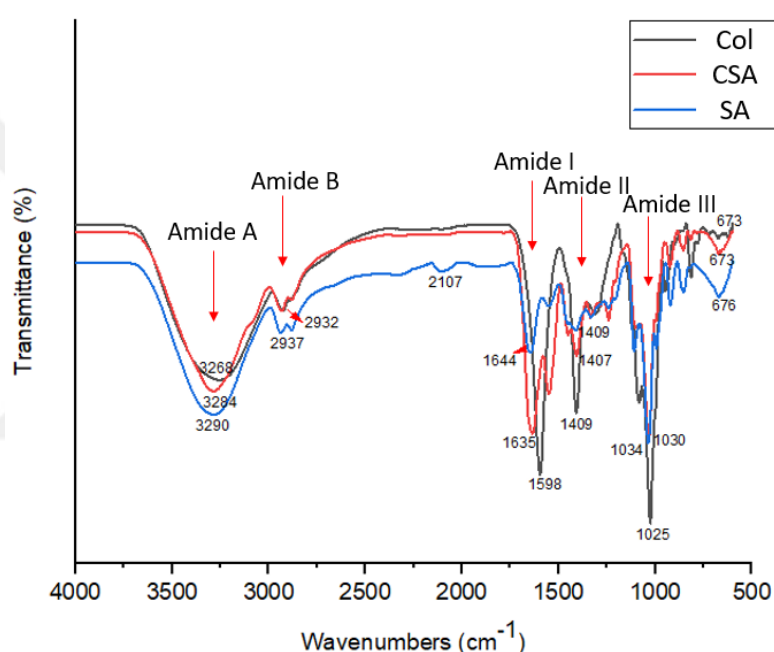


Figure 4.2: FTIR spectra of collagen film, sodium alginate film, collagen and sodium alginate composite films.

As seen in Figure 4.2, the characteristic peaks of sodium alginate films were found at 3290 cm⁻¹, 2937 cm⁻¹, 2107 cm⁻¹, 1644 cm⁻¹, 1409 cm⁻¹, 1034 cm⁻¹, 1250 cm⁻¹, 1067 cm⁻¹, 871 cm⁻¹, 749 cm⁻¹, and 661 cm⁻¹. These values line up with the research conducted by Marangoni *et al.* [226]. The hydroxyl, ether, and carboxylic functional groups all displayed significant absorption bands in the sodium alginate spectrum. At 3290 cm⁻¹, peak was observed in association with –OH stretching, were found to be similar with the results achieved in the study conducted by Pereira *et al.* [227]. The peak observed at 2937 cm⁻¹ was associated with the weak aliphatic C–H stretching

band, which was matching with the results achieved in the study conducted by Pereira *et al.* [227]. It was determined that the asymmetric and symmetric stretching vibrations of the carboxylate salt ion were responsible for the observed bands at 1644 and 1409 cm^{-1} , were found to be similar with the results achieved in the study conducted by Santos *et al.* [228]. It was determined that the bands at 1067 and 871 cm^{-1} were caused by the pyranosyl ring's C-O stretching vibration and the C-O stretching with contributions from C-C-H and C-O-H deformation.

As seen in Figure 4.2, the characteristic peaks of collagen films were found at 3268 cm^{-1} , 2932 cm^{-1} , 1598 cm^{-1} , 1409 cm^{-1} , 1025 cm^{-1} and 673 cm^{-1} . By combining four spectral intervals [(C=O) absorption of amide I, (CH₂) and (CH₃) absorptions,] (C–N), and δ (N–H) absorptions of amide III, and (C–O) and (C–O–C) absorptions of carbohydrate moieties] it was possible to achieve a reliable classification of FTIR spectra collagen film [229]. The peak found at 3268 cm^{-1} correspond to the amide A absorption band, N-H stretching and hydrogen bonding. This peak was found to be similar with the results obtained in the study conducted by Purna Sai *et al.* [220]. The peak at 2932 cm^{-1} is associated with amide B band, which was connected to an asymmetric stretch of CH₂, like the results achieved in the study conducted by Bi *et al.* [230]. That at 1598 cm^{-1} is associated with C=O stretching (Amide I), which was matching with the results achieved in the study conducted by Payne *et al.* [222]. The peak observed at 1409 cm^{-1} was associated with the CH₂ bending (Amide II), which was matching with the results achieved in the study conducted by Camacho *et al.* [231]. At 1025 cm^{-1} , peak was observed in association with C-O stretching (Amide III), parallel to the results achieved in the study conducted by Guo *et al.* [232]. The peak observed at 673 cm^{-1} was associated with the skeletal stretching.

As seen in Figure 4.2, the characteristic peaks of collagen and sodium alginate composite films were found at 3284 cm^{-1} , 2932 cm^{-1} , 1635 cm^{-1} , 1407 cm^{-1} , 1030 cm^{-1} and 673 cm^{-1} . These values line up with the research conducted by El-Rashidy *et al.* [219]. The collagen and sodium alginate composite films' FTIR spectra showed the distinctive peaks of amides I, II, and III as well as amides A and B. At 3284 cm^{-1} , peaks were observed in association with NH stretch coupled with hydrogen bending (Amide A), were found to be similar with the results achieved in the study conducted by Purna Sai *et al.* [220]. At 2932 cm^{-1} , peaks were observed in association with CH₂ asymmetrical stretching (Amide B), were found to be similar with the results achieved in the study conducted by Abe *et al.* [221]. The peak observed at 1635 cm^{-1} was

associated with the C=O stretching (Amide I), which was matching with the results achieved in the study conducted by Payne *et al.* [222]. At 1407 cm^{-1} , peaks were observed in association with CH₂ bending (Amide II), were found to be similar with the results achieved in the study conducted by Jackson *et al.* [223]. The peak observed at 1030 cm^{-1} was associated with the C-O stretching (Amide III), which was matching with the results achieved in the study conducted by Jackson *et al.* [223]. At 673 cm^{-1} , peaks were observed in association with skeletal stretching, were found to be similar with the results achieved in the study conducted by Abe *et al.* [221].

FTIR spectra of collagen and collagen and sodium alginate composite film showed similar absorbance values. It was discovered that the band's location had shifted to a lower frequency (1542 cm^{-1}) compared to the amide II band's typical absorption range (1550 cm^{-1} – 1600 cm^{-1}), which indicated that each collagen included hydrogen bonds. The asymmetrical COO stretch and OH stretch for the collagen and sodium alginate composite film proceed to a smaller wave number (from 3290 to 3284 cm^{-1}). The existence of hydrogen bonding in sodium alginate and collagen causes the wave number to change to a lower value. FTIR analysis suggests that collagen and sodium alginate most likely have strong chemical interactions.

4.2. Results of SEM-EDS Analysis of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

Morphology and elemental analysis of collagen, collagen film, sodium alginate film, collagen and sodium alginate composite film were performed by SEM and EDS analysis. The following section contains the results and analysis. Figure 4.3 presents the results obtained from the SEM-EDS analysis performed on the collagen sample.

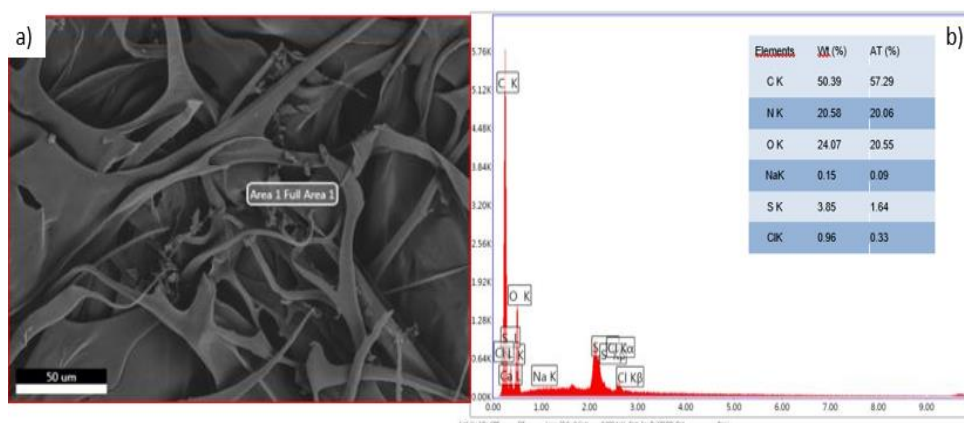


Figure 4.3: (a) SEM images (50 μm) of collagen from *Dicentrarchus labrax* scale and (b) EDS analysis of collagen from *Dicentrarchus labrax* scale.

As seen in Figure 4.3. (a), collagen exhibited a homogeneous, orderly, multi-layered and fibrillar structure. The structure of collagen, which exhibits a thin and soft sheet structure, was quite wrinkled and folded; similar exhibition with the results achieved in the study conducted by Chinh *et al.* [173]. The tissue contained collagen in the form of covalent cross-linking between different pure protein subunits, which gives the collagen its fibrous structure. [233].

The elemental composition of collagen as determined by EDS analysis is shown in Figure 4.3. (b)., collagen consisted of the elements C, O, N, S, Na and Cl. The usage of NaCl in collagen precipitation is what causes the presence of Na and Cl components [234]. The total composition of Na and Cl elements is 1.11%, which is quite low compared to other elements. Chinh *et al.* were achieve to reduce the amount of Na and Cl to 0% in their study [173]. The elements of collagen were distributed in the following order: $C > O > N > S > Cl > Na$. There was a striking similarity between the percentage weights of the components found in collagen and the findings of the investigation carried out by Chinh *et al.* [173]. In this study, the total amount of Na, Cl is low and high purity collagen is obtained, although there is some impurity issue [183].

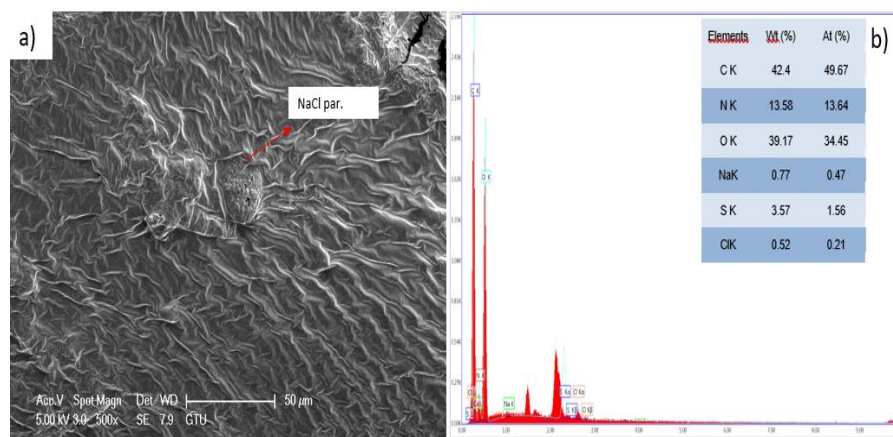


Figure 4.4: (a) SEM images of collagen film (50 µm) and (b) EDS analysis of collagen film.

Figure 4.4. (a) shows the SEM image and EDS analysis of collagen film. Collagen film matrix was found to have collagen fibers organized in a nonporous form. Although it exhibits a wavy appearance due to its regular fiber structure, it has been observed that the film contains NaCl particles used for demineralization of collagen in some parts of the film.

The elemental composition of collagen film as determined by EDS analysis is shown in Figure 4.4. (b). Collagen film consisted of the elements C, O, N, S, Na and Cl. As seen in the EDS analysis, which confirms the presence of NaCl crystals in the SEM image, there are 1.29% Na and Cl elements in the film components. It was thought to be caused by the purification issue. The elements in the collagen film were distributed in the following order, just as in the elemental distribution of collagen: C > O > N > S > Na > Cl. There was a striking similarity between the percentage weights of the components found in collagen and the findings of the investigation carried out by Chinh *et al.* [173].

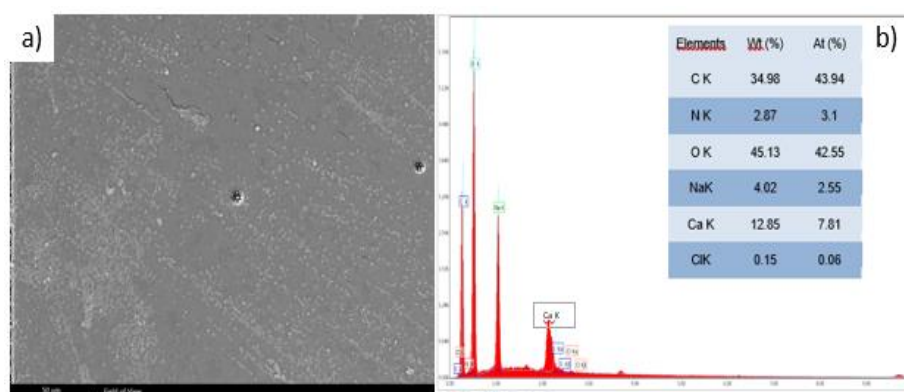


Figure 4.5: (a) SEM images of sodium alginate film (50 µm) and (b) EDS analysis of sodium alginate film.

As seen in Figure 4.5. (a), sodium alginate film exhibited a smooth surface structure with slightly homogeneous morphologies due to the dust. This SEM image is matched the sodium alginate images typically reported in the literature [235,236].

As expected, and shown in Figure 4.5. (b), carbon weight is 34.98%, nitrogen weight is 2.87%, oxygen weight is 45.13%, calcium weight is 12.85%, sodium weight is 4.02% and chlorine weight is 0.15% in sodium alginate film. These weight (%) distribution, had good match with the results achieved in the study conducted by Ostadazadeh *et al.* [236].

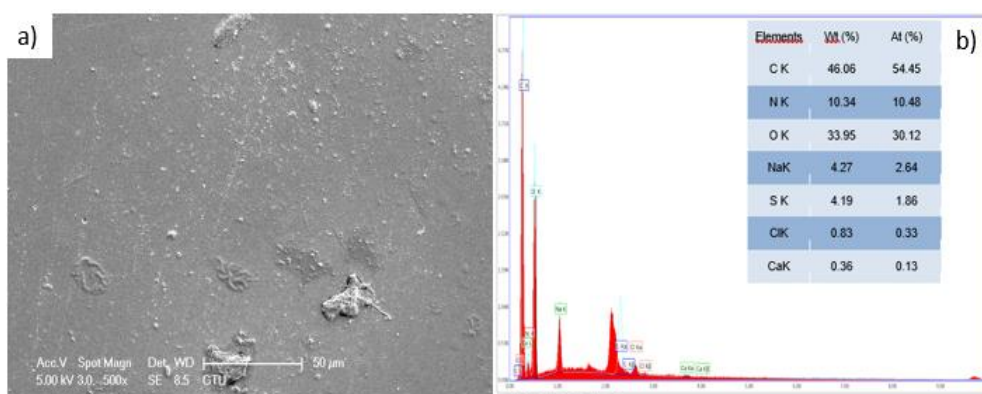


Figure 4.6: (a) SEM images of collagen and sodium alginate composite film and (b) EDS analysis of collagen and sodium alginate composite film.

As shown in Figure 4.6. (a), the collagen and sodium alginate composite film had a smooth surface in general, with visible irregularity at some points. It was

hypothesized that the dust that had accumulated on the surface may be the root cause of this irregularity. The surface of the films composed of sodium alginate had a smoother and flatter form, in contrast to the composite films comprised of sodium alginate and collagen. The SEM images were examined in conjunction with the findings that were obtained from the research carried out by Junior *et al.* [226].

According to the EDS analysis that is shown in Figure 4.6 (b), the percentages of weight of the elements in the collagen and sodium alginate composite film are as follows: the carbon is 46.06%, the nitrogen is 10.34%, the oxygen is 33.95%, the sodium is 4.27%, the sulfur is 4.19%, the calcium is 0.36%, and the chlorine is 0.83%. As seen in Figure 4.6 (b), ion exchange occurred between sodium alginate and collagen. The weight of Ca in the sodium alginate film is higher than that in the composite film, and the weight of S in the collagen film is higher than that in the composite film. This indicates that ion exchange occurs between Ca and S ions in the composite film. This change has proven successful interpenetration of alginate and collagen [236].

4.3. Results of DSC Analysis of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

For the purpose of characterizing the materials' thermal behavior, differential scanning calorimetry (DSC) was employed. DSC thermograms can reveal information about the interactions between a collagen and a sodium alginate by revealing details about peaks, such as peak appearance or disappearance or melting points.

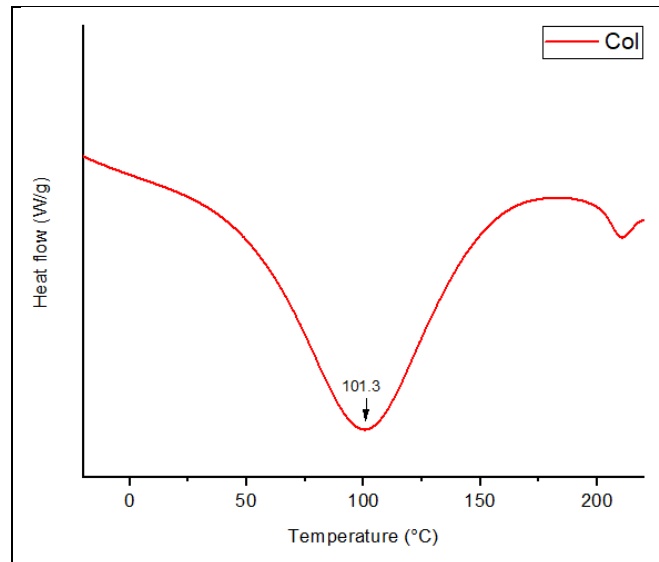


Figure 4.7: DSC thermograms of collagen.

In Figure 4.7, the endothermic peak at 101.3°C (T_g) was seen on DSC diagram from the first heating scan of freeze-dried collagen. This outcome was similar to the collagen T_g value in Héctor Capella Monson's *et al.*'s study [238]. Collagen changes from its helical configuration to its coiled state at this temperature. In the dry condition, collagen molecules are tightly packed. The fibrils of collagen are randomly fragmented as a result of heat denaturation.

In the graph of collagen film, sodium alginate film, and sodium alginate composite film are obtained as a result of the DSC analysis, the relevant peak temperatures and the thermograms of the analysis are shown in Figure 4.8.

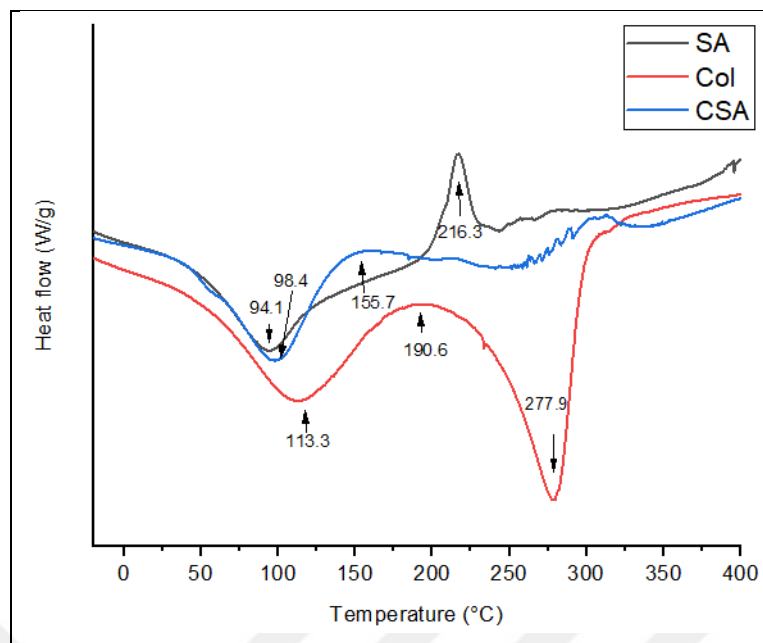


Figure 4.8: DSC thermograms of collagen, sodium alginate and composite film types.

An endothermia associated with the melting of the regular structures in the content of the films occurred at the points shown at the respective peak points. As seen in Figure 4.8, the T_g value of the collagen film is 113.3, the T_g value of the sodium alginate film is 94.1, and the T_g value of the composite film is 98.4. The fact that the T_g value of the collagen film is higher compared to the other film compositions indicates that the thermal stability is decreased with the addition of sodium alginate. This behavior in thermal stability coincides with Wang *et al.* study in the literature [237]. That can be explained by the fact that the breakdown of the collagen structure took place at lower temperature. The addition of sodium alginate caused the triple helix structure of collagen to open [239]. Nonetheless, as the SA concentration increased the T_g value for the CS films first increased and subsequently dropped. The hydrogen linked networks and water molecules are responsible for the collagen film's stated T_g value [83].

As seen in Figure 4.8, the T_c value of the sodium alginate film is 216.3, the T_c value of the collagen film is 190.6, and the T_c value of the composite film is 155.7. A third peak was observed at 277.9 in the collagen film, which was thought to be due to the carbonization temperature of the sample. The T_c value of the composite film is lower than sodium alginate film and collagen film. A low T_c value was associated with

reduced weak bonds such as hydrogen bonding between the matrix and water molecules. With the addition of sodium alginate, the hydrophobic feature has been added to the collagen and the evaporation of water has been facilitated. With the addition of sodium alginate, the thermal properties of the composite film were improved.

4.4. Results of TGA Analysis of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

Sample's weight change analyzed with TGA device. The temperature was increased regularly in the range of 25 °C to 800 °C and the change in sample weights was observed. After the samples were placed in the instrument, the balance was put in place, the temperature was started to be increased and the changes in the weight were recorded accordingly. Phase transitions occur at points where the weight of the sample changes.

The maximum decomposition ($T_{\max}$) and initial degradation (T_{onset}) temperatures for all of the samples recorded on the thermograms are shown in Table 3.1.

Table 3.2: Initial degradation (T_{onset}) and maximum decomposition ($T_{\max}$) temperatures of the samples.

Sample	First thermal event (°C)		Second thermal event (°C)		Third thermal event (°C)	
	T_{onset}	$T_{\max}$	T_{onset}	$T_{\max}$	T_{onset}	$T_{\max}$
Collagen	21	45.2	268.7	310.2	--	--
Sodium alginate film	28.2	62.3	180.7	218.5	--	--
Collagen film	31.6	88.2	152.6	235.3	316.4	356.7
Collagen and sodium alginate composite film	22.5	46.7	120.8	175.4	247.7	290.4

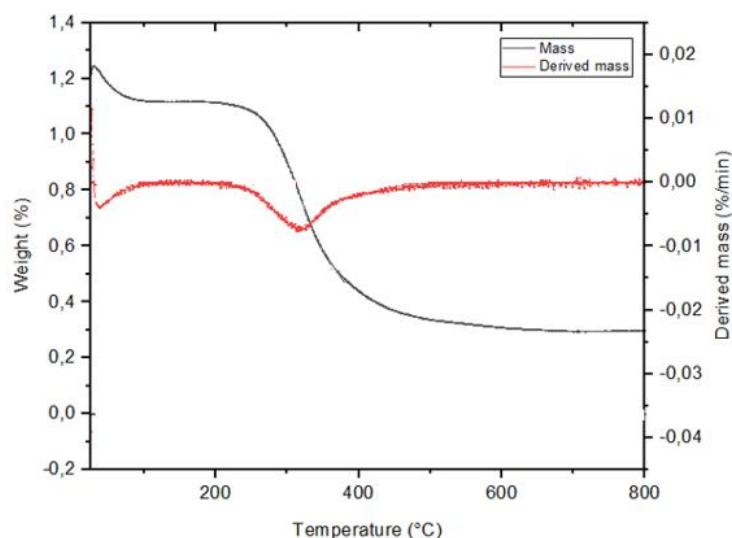


Figure 4.9: TGA and DTGA curves of collagen.

As can be seen in the graph of the thermogram and its derivative in Figure 4.9, it formed a peak corresponding to 10% of the total mass at approximately 32.9 °C, due to the loss of water and residue in the collagen. According to Correia *et al.*, mass changes up to 200°C occur in relation to the different forms of interaction that water enters with the collagen structure [240]. This change is associated with the triple helix structure of collagen and through hydrogen interactions between water and protein chains. The next degradation step occurred between 240°C and 500°C ($T_{\text{onset}} = 268$ °C). Mass loss in this range was associated with degradation of collagen and consequent loss of amino acids. Collagen derived from *Dicentrarchus labrax* scale had good thermal stability at temperatures under 200°C, as shown in Figure 4.9.

Figure 4.10 shows the mass loss through thermal decomposition of different film forms and their associated derivatives.

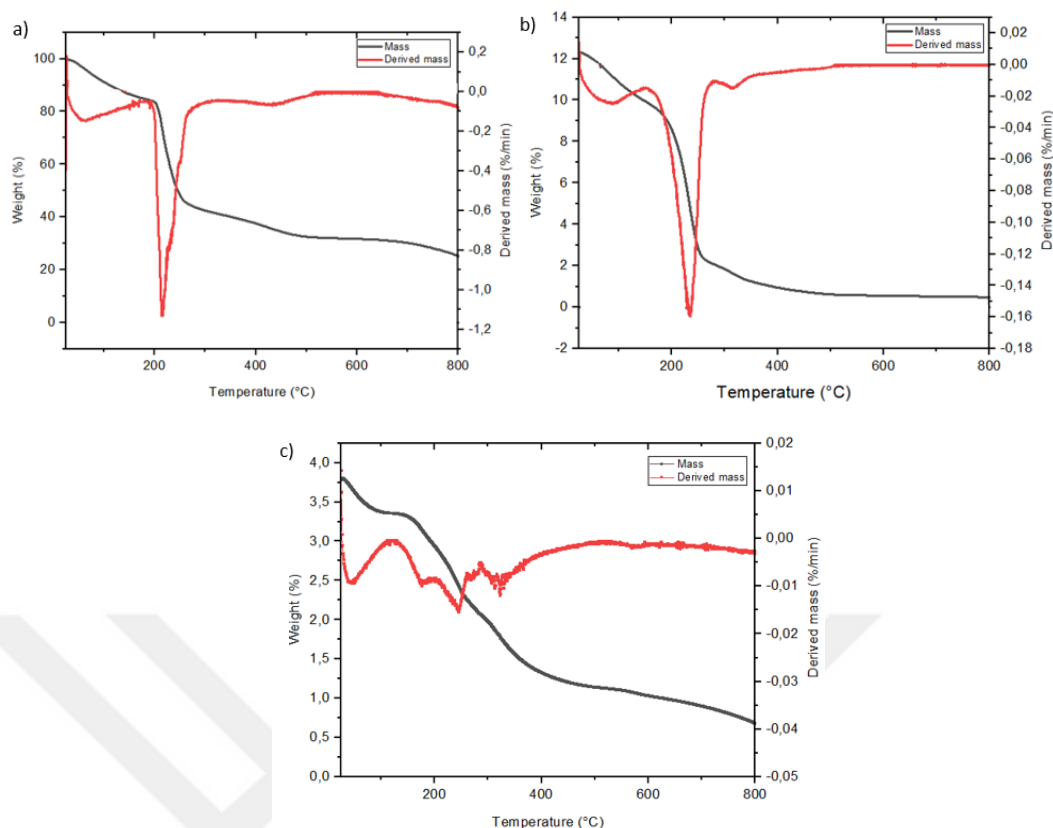


Figure 4.10: TGA and DTGA curves of (a) sodium alginate film (b) collagen film (c) collagen and sodium alginate composite film.

As can be seen in the thermogram and their derivative in Figure 4.10(a), two different thermal events occurred in the sodium alginate film. The first thermal event occurred before 100°C, which was associated with the release of water from the film [235]. The second thermal event resulted from the decomposition of the sodium alginate components [241]. As seen in Table 3.1, the T_{onset} and T_{max} for the sodium alginate film were 214.37 and 226.51 °C, respectively. These results are consistent with the results of the study by Aloui *et al.* [235].

As can be seen in the thermogram and their derivative in Figure 4.10(b), three different thermal events occurred in the collagen film. First stage weight loss ($WL_1 = 18.9\%$) in the collagen film was observed at the initial temperature (T_{onset}) of 31.6 °C, and this stage was associated with water loss from the film [240]. The second stage weight loss (48%) occurred at the initial temperature of 152.6 °C (T_{onset}). Weight loss at this stage was associated with structurally bound water loss, as well as glycerol used as a plasticizer and smaller proteins in the compound. The weight loss (15.7%) that developed in the third stage was observed at the initial temperature of 316.4 °C.

According to Tian *et al.*, between 300°C and 500°C, mass loss due to protein degradation occurs due to the release of nitrogen as ammonia and the formation of amino carbon residues [242].

As can be seen in the thermogram curve in Figure 4.10(c), the first thermal event occurred at the initial temperature (T_{onset}) of 22.5 °C (10.19% of weight loss), which was associated with the release of water from the collagen and sodium alginate composite film [122]. The second thermal event occurred in (T_{onset}) of 120.8 °C (25.28% of weight loss), resulted from the decomposition of the sodium alginate components [130]. The third thermal event occurred in (T_{onset}) of 247.7°C (25% of weight loss), resulted from the degradation of collagen and consequent loss of amino acids. These results are consistent with the results of the study by Aloui *et al.* [235]. The next degradation steps occurred between 290°C and 600°C. Mass loss in this range was associated with degradation of collagen and consequent loss of high molecular weight of amino acids. At the final stage of thermal degradation, there was a weight loss of 18.3% of the collagen and sodium alginate composite film.

As a result of the composite film obtained by adding collagen to sodium alginate, the T_{onset} and T_{max} values of sodium alginate films (Table 1) increased. This behavior can be attributed to the increased extent of hydrogen bonding between collagen and sodium alginate. Furthermore, the collagen load increased the mean mass loss rate of the sodium alginate films (Fig. 3a); this suggests that the addition of collagen contributes to the integrity of the films at lower temperatures.

4.5. Results of XRD Analysis of Collagen, Collagen Film, Sodium Alginate Film, and Sodium Alginate Composite Film

XRD analysis was performed to obtain information about the crystal structures of the collagen, collagen film, sodium alginate film, and sodium alginate composite film. Thus, the atomic and molecular order in the sample could be examined. First, the samples were placed and fixed in the sample holder included in the instrument; After adjusting the measurement parameters such as angle of incidence and X-ray source, scanning was performed.

The diffraction profile of all films was similar with XRD peaks in the 2θ ($3^\circ < 2\theta < 40^\circ$) range.

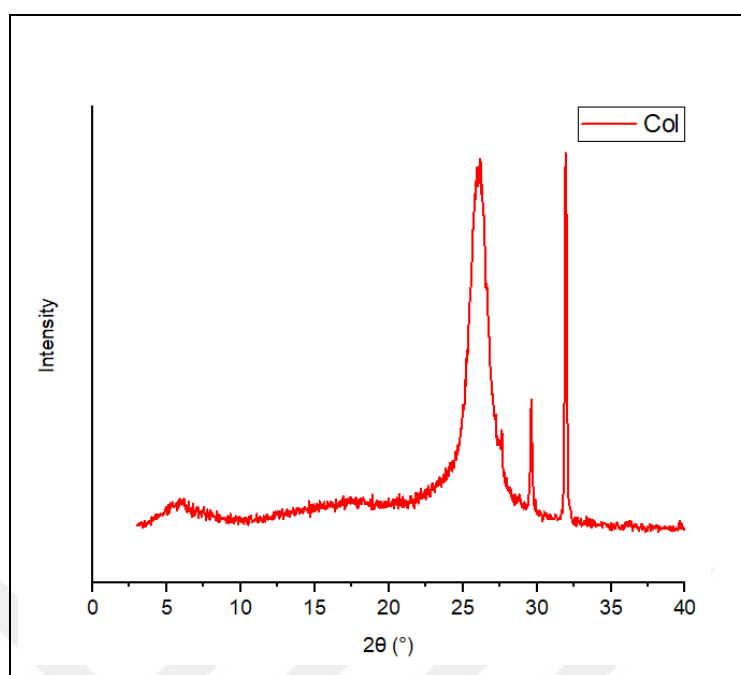


Figure 4.11: XRD pattern of collagen.

Collagen from fish scale first gave a peak around $2\theta = 6^\circ$ indicating the distance between molecular chains, then a peak at $2\theta = 27^\circ$ and third at $2\theta = 32^\circ$ the peak characteristic of the triple helix structure of collagen was seen. The small peak shown in position $2\theta = 29^\circ$ matches the triple helix structure's usual unit height [243]. These collagen-specific peaks paralleled the study by Nishihara *et al.*, confirming the triple helical structure of collagen [244].

The diffraction profile of all film types was similar with XRD peaks in the 2θ ($10^\circ < 2\theta < 80^\circ$) range.

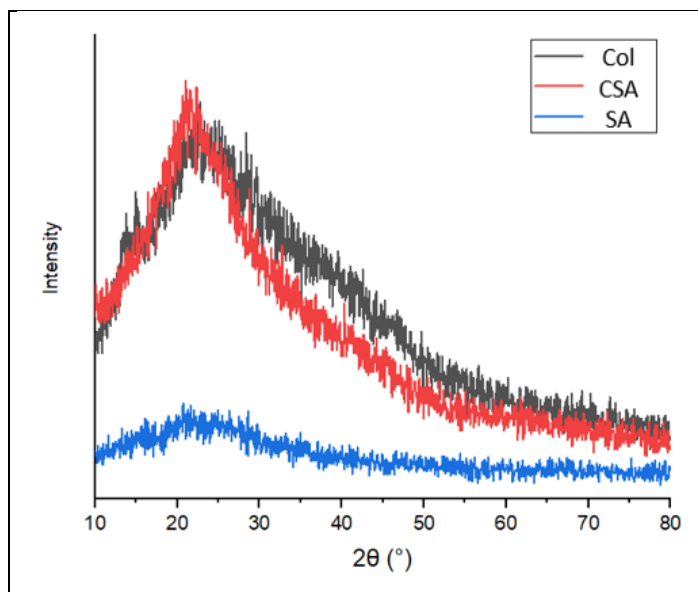


Figure 4.12: XRD pattern of collagen film, sodium aginate film, collagen and sodium alginate composite film types.

The sodium alginate films presented semi-crystalline structures, predominantly amorphous, as seen in Figure 4.12. The sodium alginate films' XRD spectra revealed a narrow diffraction peak at $2\theta = 21^\circ$, which demonstrated to low crystallinity and amorphous scatter [245].

The collagen film demonstrated diffraction peaks at $2\theta = 21^\circ$ and $2\theta = 29.6^\circ$. The two diffraction peaks that were discovered matched up with collagen's distinctive peaks [246]. The nature of the collagen component caused the broad peak at $2\theta = 21^\circ$ to have an amorphous distribution. The triple helix structure's usual unit height is represented by the modest peak at $2\theta = 29.6^\circ$ [243].

The disappearance of the small peak of collagen film at $2\theta = 29.6^\circ$ in the composite film formed after sodium alginate is added to the collagen means that the triple helix structure of the collagen is partially disrupted during dissolution.

Collagen film, sodium alginate and composite film also had a similar spectrum. However, there was an increase in the density of the crystal peaks of the composite film obtained by adding sodium alginate to collagen. In sodium alginate to collagen composite films, the diffraction peak increased due to collagen. This showed that the molecular interaction between sodium alginate and collagen was established. This suggests that the interaction between sodium alginate and collagen promotes the structural organization that increases sodium alginate crystallinity. The results were

parallel to the results achieved in the study conducted by Fabia *et al.* [247]. The molecular interaction between sodium alginate and collagen was also confirmed by the shift in vibrations of the functional groups found in the films observed in the FTIR spectrum (Figure 4.2.). It can also be associated with the increase in thermal stability with the addition of collagen to sodium alginate.



5. CONCLUSION

In this thesis study, collagen was successfully extracted from *Dicentrarchus labrax* scales and collagen film, sodium alginate film, sodium alginate and collagen composite film were successfully developed. The triple helix structure of the collagen isolated from *Dicentrarchus labrax* was confirmed by the ratio of absorption densities between the 1250 cm^{-1} (Amid III) and 1448 cm^{-1} bands, which was approximately equal to 1.0. FTIR spectra of different types of films demonstrated characteristic peaks of amides I, II and III, amide A and B. For collagen and sodium alginate composite film, the asymmetrical COO stretch and OH stretch shift to a lower wave number (from 3290 cm^{-1} to 3284 cm^{-1}). The shift of the wave number to a lower value is due to the presence of hydrogen bonds in collagen and sodium alginate. Apart from this, it was observed that there was no other bond. The triple helix structure of collagen was confirmed by the XRD analysis. The two diffraction peaks were found to correspond to two distinct collagen peaks. In addition, the XRD analysis results of the composite film consisting of sodium alginate and collagen confirmed the FTIR analysis data and showed that it interacted with molecules based on the increase in crystallinity. The resulting collagen exhibited a fibril structure. EDS analysis was performed to investigate the penetration of sodium alginate into collagen and the results confirmed the presence of sodium alginate and collagen-related elements in composite films. Its stability at temperatures below 200°C was demonstrated by the thermal analysis.

It was feasible to conclude that this product, obtained by successfully transforming collagen from fish scale into composite film with sodium alginate, may be an important candidate for use in wound healing applications. According to the findings of the studies, it has the potential to be an excellent future alternative to collagen that is utilized in the commercial sector in terms of the generation of a natural source and the evaluation of waste. To improve the composition and incorporation of additional bioactive components in the composite film, more study is still required. Clinical trials and long-term researches are required to evaluate the film's efficacy, safety, and compatibility with the human body.

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BIOGRAPHY

Melike Keskin graduated from the Department of Biomedical Engineering, in the Faculty of Engineering and Architecture, at İzmir Katip Çelebi University with a B.Sc. degree in 2020. Following her graduation, she started working in the quality assurance department of a consultancy firm that provides services for CE certification, system certification and testing of medical devices. She still continues to work here. As of fall 2020, she started her master's degree in Nanoscience and Nanoengineering at Gebze Technical University, Institute of Nanotechnology.

