Hacernur Uzuner

M.Sc. Thesis

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FABRICATION AND CHARACTERIZATION OF HEMOSTATIC CHITOSAN/GELATIN CRYOGEL CONTAINING *VERBASCUM THAPSUS* EXTRACT AND INVESTIGATION OF HEMOSTATIC EFFECT

M.Sc. THESIS

SUBMITTED TO THE DEPARTMENT OF BIOENGİNEERİNG

AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE

OF ABDULLAH GUL UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

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ACCEPTANCE AND APPROVAL

M.Sc. thesis titled “Fabrication and Characterization of Hemostatic Chitosan/Gelatin Cryogel Containing Verbascum Thapsus Extract and Investigation of Hemostatic Effect” and prepared by Hacernur Uzuner has been accepted by the jury in the Bioengineering Graduate Program at Abdullah Gül University, Graduate School of Engineering & Science.

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(Date)

Graduate School Dean
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ABSTRACT

FABRICATION AND CHARACTERIZATION OF HEMOSTATIC CHITOSAN/GELATIN CRYOGEL CONTAINING *VERBASCUM THAPSUS* EXTRACT AND INVESTIGATION OF HEMOSTATIC EFFECT

Hacernur UZUNER
MSc. In Bioengineering
Advisor: Assoc. Prof. İsmail Alper İŞOĞLU
January 2025

Hemorrhage is one of the biggest threats to human life and it causes approximately 40% of trauma deaths. To control bleeding, hemostatic dressings combining different polymers have gained interest in recent years. In this study, chitosan/gelatin hemostatic cryogels containing *Verbascum thapsus* was fabricated, characterized morphologically, chemically, biologically, and investigated hemostatic activity in vitro. Cryogels having unique porosity, rapid absorption and cell infiltration characteristics are suitable for hemostatic applications. The combination of chitosan and gelatin creates a matrix enhancing platelet adhesion and aggregation via functional groups, whereas the use of *V. thapsus* extract increases bioactivity to accelerate clot formation and shorten coagulation time. The interconnected macroporous structure, and average pore diameters of 225-478 μm were observed by SEM. The cryogels showed high swelling ratio of 3350%. The *V. thapsus* containing cryogels demonstrated the bacterial inhibitions up to 89% against *E. coli*, and 78% against *S. aureus*. The cell viability of the cryogels was investigated by a human fibroblast cell line. The blood compatibility of cryogels was proved with the 1% hemolysis ratio. The blood hemostatic activity of *V. thapsus* extract was investigated by in vitro whole blood clotting assay. The blood clotting index (BCI) of the chitosan/gelatin cryogels was improved from 11.9 to 6.5 with the addition of *V. thapsus* extract and the clotting time was decreased. The *V. thapsus* extract containing chitosan/gelatin cryogels demonstrated great hemostatic potential for hemorrhage applications.

Keywords: Chitosan, gelatin, hemostatic, Verbascum thapsus, cryogel

ÖZET

VERBASCUM THAPSUS ÖZÜTÜ İÇEREN HEMOSTATİK KİTOSAN/JELATİN KRİYOJEL ÜRETİMİ, KARAKTERİZASYONU VE HEMOSTATİK ETKİSİNİN İNCELENMESİ

Hacernur UZUNER
Biyomühendislik Ana Bilim Dalı Yüksek Lisans
Tez Danışmanı: Doç. Dr. İsmail Alper İŞOĞLU
January 2025

Kanama, insan yaşamı için en büyük tehditlerden biridir ve travma ölümlerinin yaklaşık %40'ına sebep olur. Farklı polimerlerin kombinasyonundan oluşan yeni ve biyoaktif hemostatik biyomalzemeler son yıllarda büyük ilgi görmeye başlamıştır. Bu çalışmada, *Verbascum thapsus* özütü içeren hemostatik kitosan/jelatin kriyojel üretilerek, morfolojik, kimyasal ve biyolojik olarak karakterize edilmiştir ve in vitro araştırılmıştır. Kriyojeller özgün gözenekli yapısı, hızlı sıvı absorpsiyonu ve hücre infiltrasyon özellikleri nedeniyle hemostatik uygulamalar için oldukça uygundur. Kitosan ve jelatin fonksiyonel grupları aracılığıyla trombosit yapışmasını ve agregasyonunu artırırken, *V. thapsus* özütünün kullanımı, içeriğindeki bileşenlerle biyoaktivitesi artarak pıhtı oluşumunu hızlandırarak kanama süresini kısaltır. SEM ile 225 ile 478 μm arasındaki ortalama gözenek çaplarına sahip, birbirine bağlı, makro gözenekli yapı gözlemlendi. Kriyojeller %3500'lük yüksek oranlarda şişme gösterdi. *V. thapsus* içeren kriyojeller, *E. coli*'ye karşı %89'a ve *S. aureus*'a karşı %78'e kadar bakteriyel inhibisyon gösterdi. Kriyojellerin hücre canlılığı bir insan fibroblast hücre hattı ile test edildi. Kriyojellerin kan uyumluluğu %1'lik hemoliz oranı ile kanıtlandı. *V. thapsus* özütünün hemostatik aktivitesi in vitro tam kan pıhtılaşma testi ile araştırıldı. Kitosan/jelatin kriyojellerin kan pıhtılaşma indeksi (BCI), *V. thapsus* özütü eklenerek 11,9'dan 6,5'e düşürüldü ve pıhtılaşma süresi azaltıldı. *V. thapsus* özütü içeren kitosan/jelatin kriyojeller, kontrolsüz kanama uygulamaları için büyük hemostatik potansiyel gösterdi.

Anahtar Kelimeler: Kitosan, jelatin, hemostatik, Verbascum thapsus, kriyojel

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To My Family

Chapter 1

Introduction

1.1 The Skin

The largest organ in the human body is the skin, covering an area of two square meters [1]. It has three layers: epidermis, dermis, and hypodermis. The outermost layer is epidermis which protects the multilayered cellular structure. The cell layers of the epidermis consist of various cell types at different stages of differentiation, some of which are keratinocytes and melanocytes [1, 2]. The dermis consists of an extracellular matrix (ECM), which provides mechanical strength and flexibility to the skin through elastin, collagen, proteoglycan, and hyaluronic connective tissues. A large proportion of this matrix is synthesized by fibroblast cells, which are commonly found in the dermis layer. Endothelial cells, which are responsible for the neovascularization, are another common cell type [3]. The hypodermis (lowermost layer), which consists of fat tissue, is responsible for the regulation of body temperature and energy storage while connecting skin to the bones, muscles, and organs underneath [3]. The skin protects the tissues and organs, regulates body temperature, and provides a barrier structure against external influences such as UV radiation, temperature, and microbial agents [4]. Another characteristic of the skin is that the cells on its surface continuously grow and replace dead cells, resulting in regeneration. This characteristic allows for the repair of epithelial tissue damage [1].

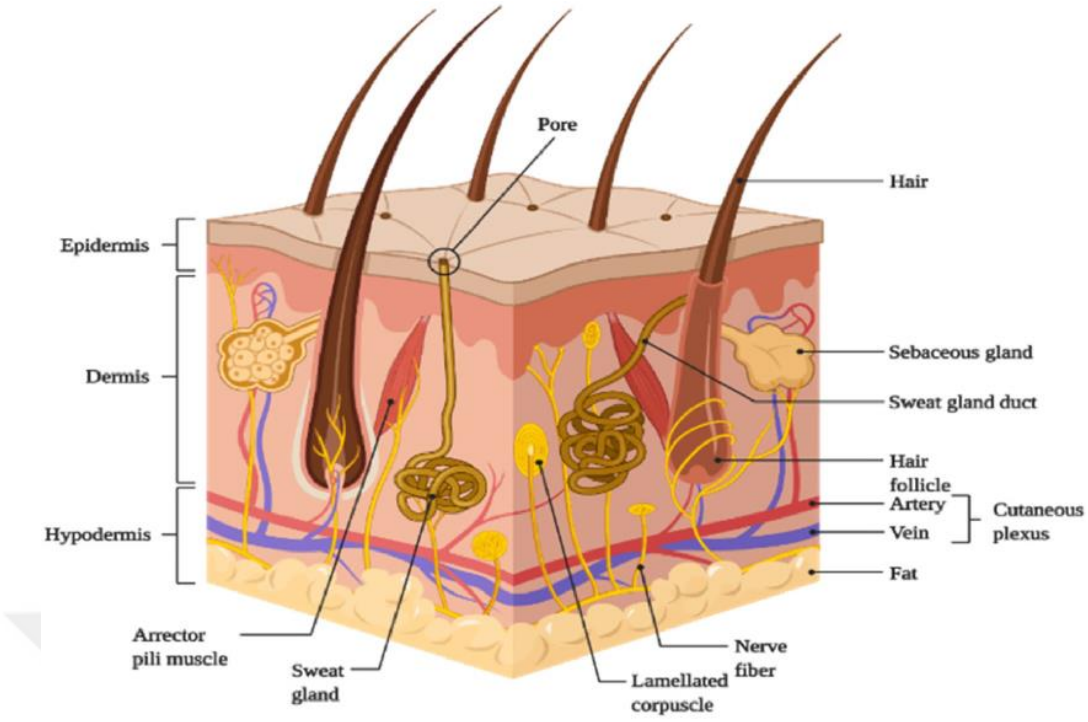


Figure 1.1 Layers of the skin [5].

1.1 The Wound and The Wound Healing

The skin is constantly subjected to external and internal factors which makes it susceptible to injuries, trauma, or burns [6], [7]. A wound is the disintegration of the anatomical structure or loss of function in the skin, muscles, tissues, nerves, blood vessels, or bone as a consequence of physical or thermal damages [6], [8]. Wound healing is composed of serial biological processes to restore tissue integrity and normal physiological circumstances of damaged tissue. Hemostasis, inflammation, proliferation, and remodeling are four phases of wound healing. Failure in any of these phases may cause the development of chronic wounds or hypertrophic scar formation [9]. Acute wounds mostly heal within 8 to 12 weeks, while chronic wounds prolong the inflammatory phase which can result in excessive exudate, microbial infections, several skin diseases, discomfort, and even tissue necrosis, so chronic wounds may take months to heal [10]. Hence, faster wound management is essential to prevent further complications.

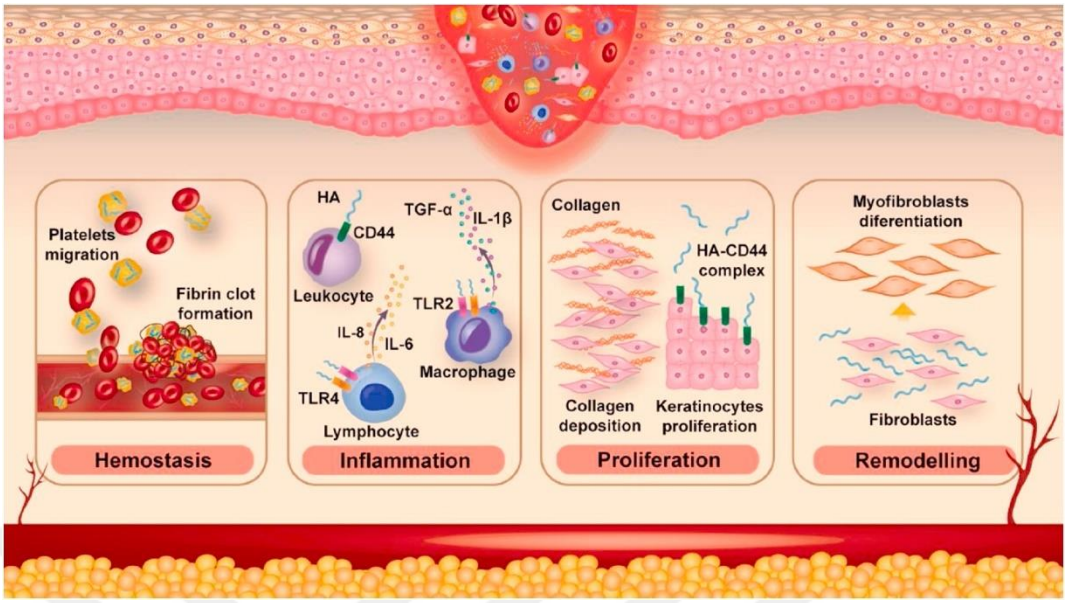


Figure 1.2 Wound healing process [11].

Hemostasis, or blood clot formation, is the human body's spontaneous and natural response to an injury in vascular structure. After an injury, the body's first response is to prevent the bleeding, which is approached through vasoconstriction by rapid constriction of smooth muscle present in the structure of the vessel wall with increased calcium level [12], [13]. Reduced blood flow leads to produce vasoactive metabolites such as nitric oxide, adenosine, acidosis, and histamine for vasodilation and increase of vascular permeability providing the transition of inflammatory cells to the wound site causing the appearance of the early wound to be warm, red, and swollen [14]. Further, blood loss is prevented by platelet activation where platelets undergo morphological changes and adhere to the sites of exposed collagen in the damaged vessels to form a platelet plug that is then reinforced by fibrin and von Willebrand factor [14]. Platelets play an important role in wound healing because they contribute to form clots and release growth factors and cytokines that induce inflammation [15]. Furthermore, the intrinsic and extrinsic coagulation pathways collaborate to create the initial platelet block. The extrinsic pathway begins when tissue factors are exposed as a result of vascular injury or trauma, which activates factor VII in the presence of calcium ions. In contrast, negative ions on the wounded tissue surface activate factor XII, which initiates the intrinsic pathway [16], [17]. Both mechanisms require a complex cascade of chemical processes that activate coagulation factors and eventually leading to the activation of factor X. Activated factor

Xa then turns prothrombin to thrombin, which converts fibrinogen into fibrin and starts fibrin polymerization at the injury site. Meanwhile, calcium ions and factor XIII help to reinforce the platelet plug by strengthening the fibrin mesh [18].

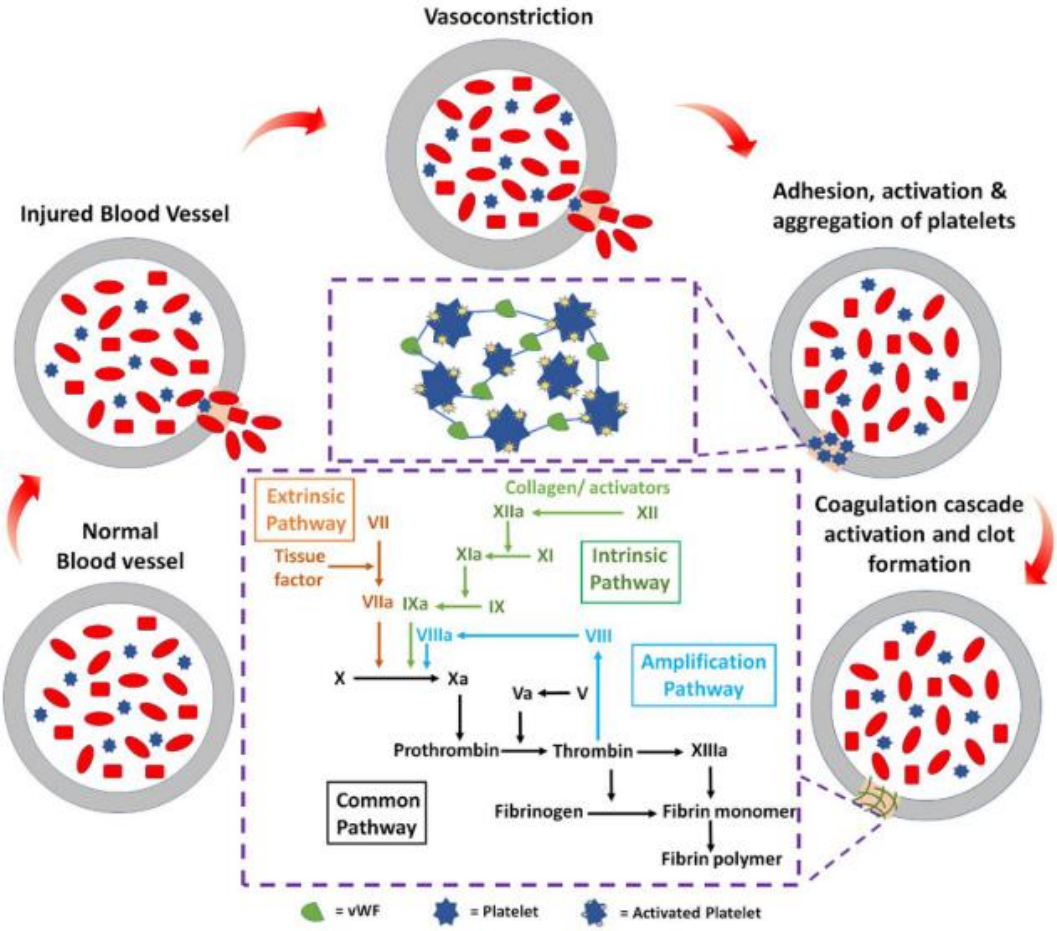


Figure 1.3 Schematic representation of coagulation cascade [19].

The inflammatory phase is the response of the immune system and aims to prevent infection. During inflammation, the neutrophils are infiltrated and migrate through several chemical signaling, such as interleukin, transforming growth factor-β (TGF-β) by chemotaxis which allows neutrophils passing towards a chemical gradient to the wound site for the first 48 hours after injury [20]. Neutrophils are responsible for the ingestion and destruction of foreign particles, cellular debris and pathogens by phagocytosis. Also, they release several toxic substances and produce oxygen free radicals as a result of neutrophil activity to destroy bacteria and dead tissues while sterilizing the wound with chlorine as well [14], [21]. Thereafter, macrophages are accumulated to the wound site by the chemical signals excreted from platelets and damaged tissue and they release a variety of growth factors which regulate wound healing, stimulate angiogenesis and

enhance granulation tissue formation. Lymphocytes also stimulate the production of ECM and collagen production for tissue remodeling [14].

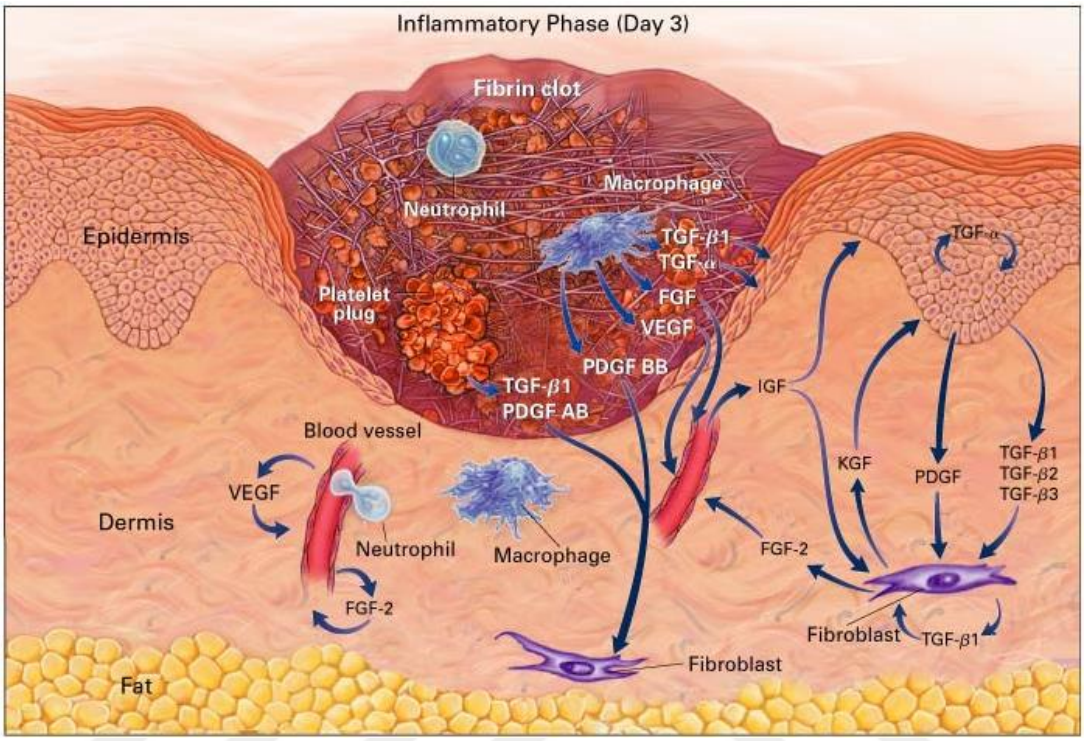


Figure 1.4 Inflammatory phase of wound healing [22].

The proliferation phase starts once the bleeding is stopped and the inflammatory response has been balanced. Once the platelet plug is formed, angiogenesis begins as platelets release growth factors like TGF-β, platelet derived growth factor (PDGF), and fibroblast growth factor (FGF). Then, vascular endothelial growth factor (VEGF) together with the cytokines promotes endothelial cells to initiate the formation of new blood vessels and repair damaged vessels. Stimulated fibroblast cells then migrate to the wound site and produce collagen and ECM proteins, such as hyaluronan, fibronectin, proteoglycans, and fibrous tissue replaces the clot which is called the granulation tissue. Then, epithelial cells cover the wound attaching to the produced ECM below. Before the epithelization, the wound contracts by myofibroblasts to decrease the area of damaged tissue to be replaced [14], [21].

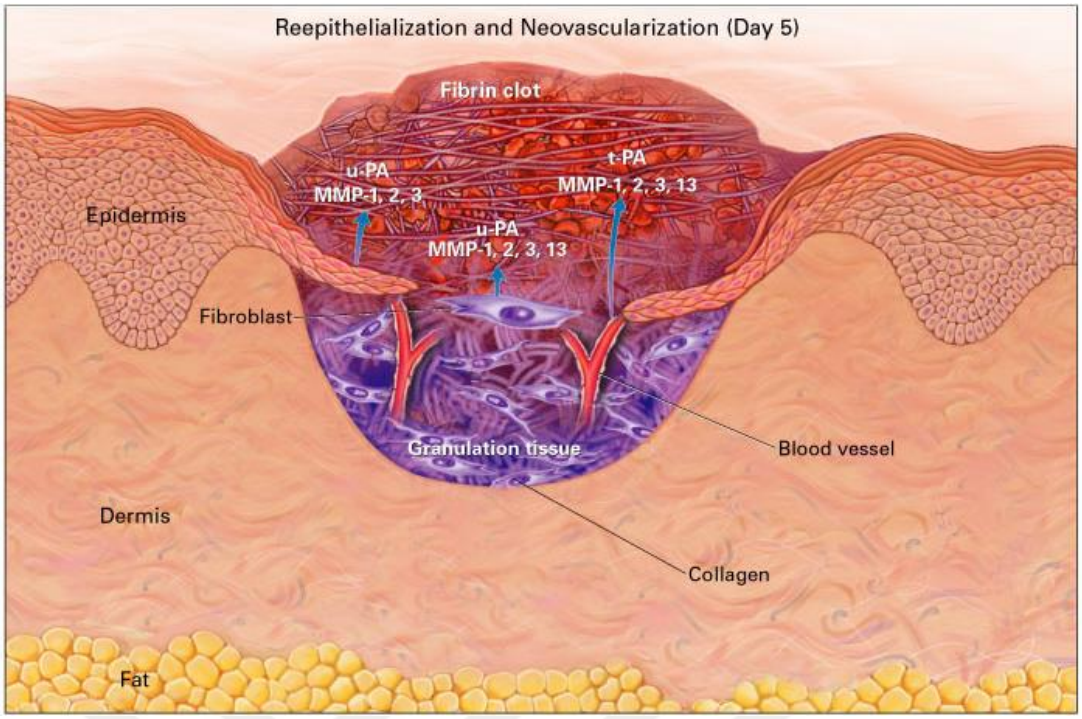


Figure 1.5 Reepithelization and neovascularization during the proliferation phase of wound healing [22].

The last phase of wound healing is remodeling which results in the formation of new epithelium. As the scar tissue is matured, synthesis and degradation activities are balanced and vascularity decreases. Prior collagen type III deposited in the ECM is replaced with collagen type I [23]. However, the mechanical strength of the wound is not fully restored but it can reach up to 80 % of the formerly tensile strength over time [14].

1.2 Biomaterials

Biomaterials are either natural or synthetic substances in contact with tissues or body fluid for the purposes of restoring, replacing, and enhancing once the tissues or organs losses its function in the body [24]. Biomaterials is a multidisciplinary field including disciplines in medicine, chemistry, physics, biology, materials science, molecular biology, mechanical engineering, and biomedical engineering [24], [25]. The design of the biomaterials varies to meet the requirements of application area. These materials are crucial in regenerative science and biomedical engineering, including the generation of prosthetics, implants, drug delivery systems, and scaffolds used in tissue engineering and wound healing [26]. Ideal biomaterials should be biocompatible as it

should be integrated into body without causing adverse immune reaction and toxicity, it should be biodegradable and should degrade into nontoxic end products which could be discarded from the body without causing harm to tissues and organs. Also, they should have mechanical strength depending on the applied tissue and should have antibacterial properties to minimize the risk of infection. Moreover, they should be easy to produce, easy to sterilize, and cost effective [25]. Natural materials like collagen, alginate, gelatin, proteins, albumin, and fibrin, also synthetic polymers such as polyvinyl alcohol and polyglycolide, as well as their mixtures in composites are often used in biomaterials [27].

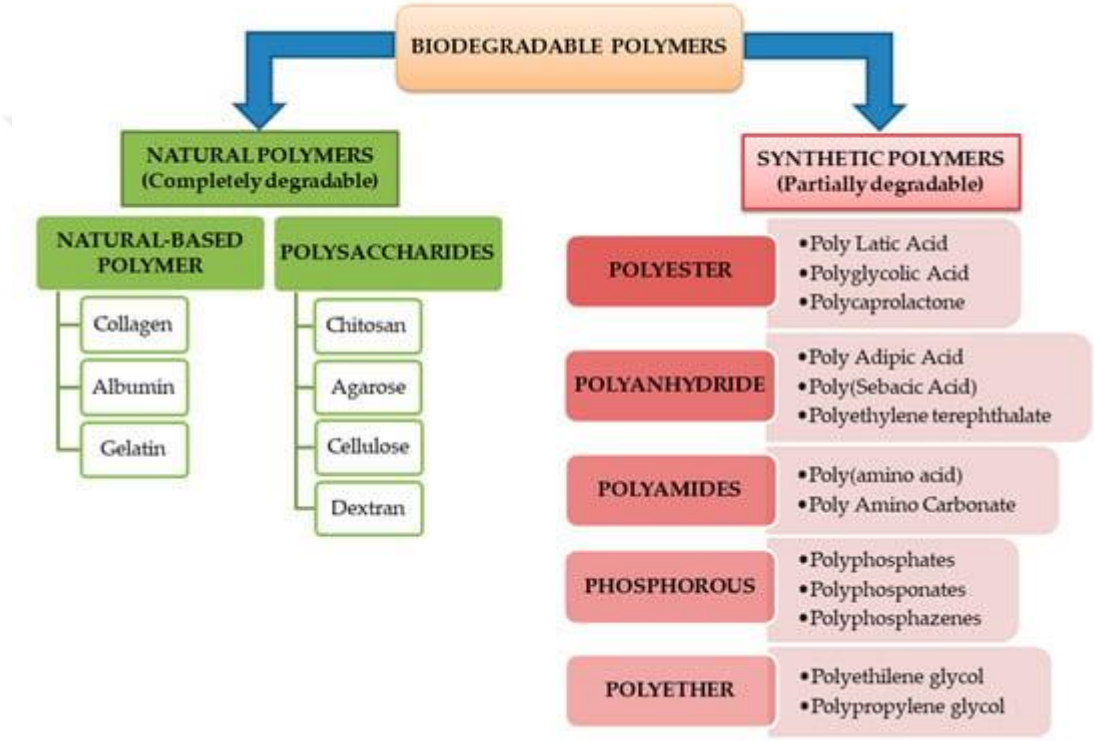


Figure 1.6 The examples of natural and synthetic polymers used in biomaterials field [28].

1.3.1 Natural Biomaterials

Natural biopolymers have been discovered as important bioactive components in therapeutic materials in recent years. Biopolymers are mostly categorized into following three types, polypeptide based or protein based (collagen, fibrin, silk, etc), polysaccharide based (chitin, chitosan, alginate, cellulose, etc), and polynucleotide based (DNA and RNA, etc.), as well as decellularized extracellular matrix [27], [29] . Natural biopolymers have various advantages, including minimal or no toxicity, increased bioactivity,

improved the response of cells, superior biocompatibility, and high hydrophilicity, which make them biologically efficient. However, they have some drawbacks, such as variability in isolates, limited stability and repeatability, poor processability and solubility, the potential of contamination, and limitations in flexibility, strength, durability, and cost-effectiveness [29].

Alginate, the negatively charged polysaccharide-based polymer, is formed by alternating the residues of mannuronic and guluronic acid [30]. It is usually obtained from brown algae using an alkaline solution [31]. Alginate is biocompatible, biodegradable, hydrophilic, and indicated as safe by the FDA (Food and Drug Administration). Alginate establishes a moist environment that minimizes bacterial infections and stimulates the release of certain cytokines accelerating the healing process. Also, alginate promotes the production of granulation tissue and re-epithelialization, which aids wound healing [32]. Alginate's biocompatibility, low toxicity, affordability, and ability to form gels in the presence of divalent cations (e.g., Ca^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}) providing highly promising material in drug delivery, scaffoldings, and wound dressing applications [33]. In a study, Ma et al. fabricated a wound dressing that combines carboxymethyl chitosan, sodium alginate, and Ce^{3+} ions to improve antibacterial characteristics and stability. The dressing showed good biocompatibility and allowed complete wound healing within 10 days in mice, with antibacterial activity against *S. aureus* and *E. coli* [34]. Iglesias-Mejuto and García-González found that 3D-printed alginate and hydroxyapatite aerogel scaffolds which had high porosity, promoted cell adhesion and proliferation, with about 100% cell viability after 48 hours [35]. In another work, Eldeeb et al. produced a composite alginate hydrogel with pitavastatin nanovesicles, which demonstrated prolonged drug release and improved wound healing. The nanocomposite hydrogel greatly improved skin regeneration in surgically generated wounds in dogs as compared to alginate hydrogels and drugs [36]. Additionally, Asadi et al. improved gauze and polyamide by incorporating with silver nanoparticles (AgNPs), poly(hexamethylene) biguanide (PHMB) and alginate using a dipping technique and evaluated the hemostatic activity of the composite dressing material. It has been observed that coagulation and water absorption capacity of the dressings were increased due to alginate polymer and antibacterial properties were enhanced by AgNPs [37]. A novel composite sponge for hemostatic purposes were produced by Zhou et al. through Ca^{2+} crosslinking and freeze-drying of gelatin and sodium alginate. The results demonstrated that gelatin/sodium

alginate sponge showed similar hemostatic time and blood loss similar to commercial sponges and enhanced by sodium alginate to promote platelet adhesion and aggregation compared to gelatin sponge [38].

Cellulose is a primary component of cell walls, a linear polysaccharide made up of 1,4 linked β-D-glucose, is the most common natural biopolymer found in plant cells, bacteria, and algae [32]. It is a biocompatible, bioactive, biodegradable, and it has adequate physical and mechanical properties due to amorphous and crystalline regions making cellulose suitable for biomaterials and scaffolding [39]. For instance, Mahendiran et al. produced porous scaffolds using a cellulose from decellularized *Borassus flabellifer* and form a hybrid of the freeze-dried cellulose and chitosan for tissue engineering. Both scaffolds exhibited good porosity (50-200 μm), and controlled degradation rate. The cellulose scaffold showed higher swelling capacity whereas hybrid scaffold showed higher cell viability and cell attachment [40]. Modified versions of the cellulose also can be used, such as nanocellulose and oxidized cellulose which has high absorption capacity, low hemolysis rate, and high mechanical strength [41]. Volz et al. produced nanocellulose scaffold from *Gluconacetobacter xylinus* for vascularized adipose tissue engineering. The scaffold exhibited accelerated cell differention of adipose derived stem cells, and comparable maintenance of adipocytes and vascular endothelial cells [42]. Also, Sun et al. developed a composite material from cellulose hydrogel and keratin-catechin nanoparticles and the results demonstrated good adhesiveness, blood cell adsorption and less blood loss with rapid coagulation in a rat model [43].

Hyaluronic acid (HA) is a highly versatile glycosaminoglycan existing in the human body and produced by fibroblasts and chondrocytes. Its unique molecular structure containing functional groups, such as alcohol, carboxylic acid, and amide allows for a wide range of chemical changes [44]. HA absorbs and retains water to support the ECM structure owing to its hydrophilic properties, and negative charge, and it is essential for cellular interactions and wound recovery [44], [45]. Numerous HA based materials such as hydrogels, electrospun fibers, scaffolds, patches, and nanoparticles have been developed broadening their clinical applications, particularly in regenerative medicine [44]. For example, Arulmoli et al. produced a hydrogel scaffold consist of salmon fibrin, hyaluronic acid, and laminin which demonstrated differentiation and proliferation of human neural stem/progenitor cells, and enhanced vessel formation where fibrin provided

structural support, HA helped with cell attachment and hydration, and laminin enhanced cellular adhesion and differentiation [46]. Choi et al. produced HA nanoparticles conjugated with PEG for drug delivery. PEGylation of the nanoparticles increased the stability and circulation time but reduced the cellular uptake by normal fibroblast cells while the cancer cells that overexpress a HA receptor called CD44 take up large amounts of the nanoparticles suggesting its potential to be used for cancer therapy and diagnosis [47]. Gao et al. investigated the hemostatic activity of HA conjugated with collagen-binding peptide and von Willebrand factor–binding peptide which was called as HAPPI. The results showed that HAPPI accumulated platelets at wound site by selectively binding to activated platelets, significantly reduced blood loss and clotting time which has potential to be used as intravenous hemostat [48].

1.3.2 Synthetic Biomaterials

Synthetic polymers, on the other hand, offer better tailoring of chemical composition, processing, and mechanical characteristics while lacking bioactivity, lower cell adherence, hydrophobicity, and restricted recognition of the cell surface [49]. The characteristics of every polymer are determined by its innate nature, production techniques, and final product designation [27].

Poly lactic-co-glycolic acid (PLGA) is a linear aliphatic copolymer formed by either by polycondensation of lactic and glycolic acid monomers, which results in low molecular weight PLGA or by ring-opening polymerization of the monomers, resulting in PLGA with higher and more consistently distributed molecular weight [50]. PLGA, a biocompatible and biodegradable copolymer, is FDA-approved, has a tunable degradation rate depending on the lactide/glycolide ratio, dissolves through hydrolytic de-esterification, and its metabolites are naturally removed by the body [51], [52]. However, due to its lack of osteoinductive qualities and poor mechanical strength, particularly in load-bearing applications, rather than using PLGA alone, it is preferable to combine it with other materials in tissue engineering applications [32]. Babilotte et al. fabricated 3D printed porous scaffold from PLGA combined with hydroxyapatite nanoparticles to overcome the osteogenic limitations of the PLGA scaffold. The scaffolds were highly cytocompatible, with 100% cell viability in both human adipose-derived stem cells and human bone marrow stromal cells. After 14 days, alkaline phosphatase (ALP)

was observed, followed by substantial calcium deposition after 21 days, showing osteogenic potential. The scaffolds' *in vivo* biocompatibility was assessed by implanting them subcutaneously in Wistar rats and histopathological examination after implantation indicated a minimal inflammatory response, showing that the scaffolds are biocompatible[53]. Petri et al. fabricated a composite scaffold from PLGA mesh and collagen sponge for cartilage tissue regeneration. the scaffold showed high porosity presenting interconnected both large and small pores ranging from about 50 nm to 440 nm, which provided mechanical strength and promoted bovine articular chondrocyte proliferation after 7 days. After 8 weeks of *in vivo* implantation in nude mice, homogeneous cell distribution and proliferation within the scaffold were observed, as well as uniform cartilaginous matrix formation [54]. Moreover, Yuan and Liu developed a gauze loaded with granulocyte colony-stimulating factor (G-CSF) containing dextran nanoparticles coated with PLGA through spraying onto scaffold and vacuum dried aiming post-surgical wound healing. It was demonstrated that along with the PLGA coating and the G-CSF nanoparticles, the scaffolds showed enhanced hemostasis while tissue regeneration and accelerated wound healing was also observed due to the controlled release of G-CSF [55].

Polycaprolactone (PCL) is a biodegradable, semi-crystalline polyester synthesized through various methods such as polycondensation of a hydroxycarboxylic acid, and the ring opening polymerization of a lactone with ranging from 14.000 to 100.000 molecular weight [56]. PCL has suitable toughness and mechanical strength at physiological temperatures due to melting point (about 60°C) and glass transition temperature (-60°C) and it has the slowest degradation rate among polyesters due to its structure including repeating hydrophobic -CH_2 groups and the degradation rate depends on alterations in its crystallinity, molecular weight, and degradation conditions [32], [57]. PCL has biocompatibility, adjustable biodegradability, strength, ease of synthesis, adaptability in shape, minimal acidic byproducts, and high drug permeability allowing its usage in tissue engineering applications and it is approved by FDA [57], [58]. However, struggles such as slow degradation rate, limited cell adhesion, and non-osteogenic characteristics have given rise to the development of PCL-based composites combining other biomaterials [32]. In a study, Kumar et al. developed a scaffold made of PCL, polyglycolic acid (PGA), and beta-tricalcium phosphate through solvent casting and particulate leaching demonstrating suitable porosity. The increase in mechanical strength (about 92 MPa) and

decrease in degradation rate about 40% was achieved with blending PGA while bioactivity was provided by beta-tricalcium for bone regeneration [59]. Ghalia and Alhanish developed a composite porous scaffold combining PCL with polyethylene glycol (PEG) and integrating cellulose nanofibers soft tissue engineering applications. The results exhibited that the addition of PEG the degradation rate was increased while providing mechanical integrity, cellulose nanofibers enhanced the tensile strength, and the adequate porous structure was observed for cell infiltration ensuring the scaffold could be used for tissue regeneration purposes[60]. PCL has also been used as a tissue sealant for hemostatic purposes. For example, Zhang et al. produced a composite scaffold composed of PCL in between the layers of gelatin and chitosan which demonstrated suitable degradability, biocompatibility and cell attachment for tissue regeneration in addition to accelerated hemostasis [61]. Also, Park et al. fabricated a nanofibrous wound dressing made of electrospun PCL and calcium carbonate coated by sprayed β-chitosan for hemostatic purposes. The results demonstrated that the dressing showed better hemostatic performance compared to PCL nanofibers due to improved surface wettability by β-chitosan and accelerated coagulation by calcium carbonate [62].

Polyethylene glycol (PEG) is a flexible, biocompatible polyether produced from petroleum that has a broad application in tissue engineering and medical applications [41]. Because of its minimal toxicity and immunogenicity, PEG is considered as a good biomaterial for biomedical applications. Its molecular weight and structure can be adjusted to influence its solubility, degradation rate, and interactions, allowing for more specialized uses. PEG's hydrophilicity improves drug delivery and bioconjugation. It degrades into non-toxic metabolites, hence alleviating potential safety issues [63]. In a study, Vallmajo-Martin et al. designed a hybrid hydrogel consisting of transglutaminase (TG) crosslinked system combining PEG and HA with the goal of developing bone marrow organoids that are both physiologically and mechanically adjustable. The PEG/HA incorporation allowed to tailor the hydrogel's features, such as stiffness and degradation rate, to better simulate the natural bone marrow conditions. The findings revealed that the organoids facilitated stem cell proliferation and differentiation, making them a suitable structure for bone marrow tissue engineering and testing for medications [64]. In another study, Elgamal et al. developed a nanocomposite material of PEGylated carbon nanotubes as a carrier for ixazomib, a drug used to treat myeloma. The PEG coating improved the biocompatibility of the drug and stability of carbon nanotubes,

resulting in better drug delivery to cancer cells. The composite showed effective drug release and increased cellular uptake by cancer cells, indicating the possibility for enhanced treatment in myeloma due to targeted delivery and controlled release of the ixazomib [65]. It also has been used in hemostatic biomaterials. Lewis et al. produced collagen pads coated by PEG to achieve effective hemostasis for post-surgical applications. The results showed that the materials had rapid swelling ability, self-adhering ability to the tissues and provided good hemostatic effect in a heparinized porcine pulmonary segmentectomy model supporting its potential for bleeding control and wound sealing [66]. Lih et al. produced chitosan hydrogels grafted via PEG modified by tyramine to enhance solubility that could quickly solidify after application to control bleeding and enhance wound healing. The results demonstrated that in situ curable hydrogels showed suitable tissue adhesiveness, and hemostatic efficiency in liver defect model in rat compared to fibrin glue and other control groups along with superior wound healing properties [67].

1.4 Hemostatic Biomaterials

Blood has many important roles in the human body. In addition to carrying nutrients and oxygen to all tissues and organs in our body, it also regulates many mechanisms in the body [68]. Almost 1.9 million of people die every year due to hemorrhage [69]. Excessive and uncontrolled bleeding mostly occur because of medical operations, surgeries, diseases, traumatic injuries [70]. Moreover, hemorrhage causes the 30-40 % of the traumatic deaths [41]. Also, loss of 40% of the blood by volume result in death [71]. When severe bleeding exceeds the capability of the hemostasis process, hemostatic agents or hemostatic biomaterials are required to accelerate coagulation and control the bleeding.

Hemostatic biomaterials are particular materials that enhance hemostasis by accelerating blood clot formation, stimulating clotting factors, or functioning as physical barriers. They have a vital role in trauma care, surgery, and wound management to rapidly control and prevent excessive bleeding [72]. The ideal hemostatic biomaterial should control bleeding effectively and quick, sustain hemostasis for several hours, should be removed easily without leaving residues, should be easy to produce and sterilize at low

cost, should be easy to store, apply and should be portable. Lastly, hemostatic materials should have high hemocompatibility and biocompatibility without causing side effects on healing and further complications such as thromboembolism [73].

1.4.1 Mechanism Action of Hemostatic Biomaterials

Hemostatic materials achieve hemostasis through various mechanisms. The hemostatic materials can be design depends on the criteria of absorption of blood plasma, physical adhesion to injured tissue, or chemical interactions with coagulation factors to initiate coagulation cascade [74]. The traditional hemostatic materials like gauze, tourniquets, and sponges absorb the blood to control excessive bleeding, however they cannot meet the requirements of completely hemostasis, and tend to unwanted adhesion on the wound site leading delayed wound healing as well [75], [76]. The second approach is the controlling bleeding by physical adhesion, the bio-adhesive materials can use to adhere on the bleeding area physically. However, the strong the bio-adhesive materials are obtained from cyanoacrylate based synthetic polymers having disadvantages such as toxicity, triggering immune responses, and causing exothermic polymerization with tissues resulting in tissue burns and increased stiffness [77]. The last approach is combining with hemostatic agents to trigger and accelerate coagulation [74]. These hemostatic agents are minerals activating coagulation cascade by interactions of surface charges, silica, whitlockine and silicates such as zeolite, kaolin, laponite, and montmorillonite [74], [78], [79]. However, these hemostatic bioactive agents have potential risk to be released into vascular circulatory system leading to the undesired clot formation throughout blood vessels. Therefore, it’s important to design the hemostatic materials with controlled and efficient hemostatic activity without unwanted side effects.

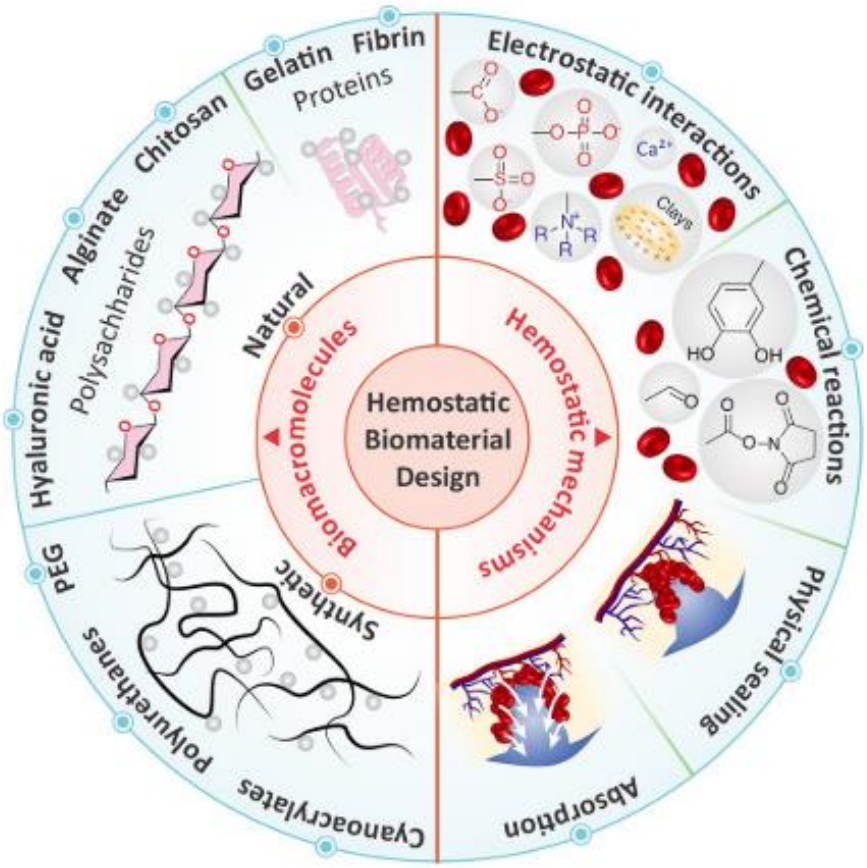


Figure 1.7 Design approaches of hemostatic biomaterials [16].

1.4.2. Types of Hemostatic Materials

Hemostatic materials aiming for blockage of blood flow and rapid blood clotting in different forms are being developed with biomaterial approaches [74]. Conventional hemostatic materials like gauze, tourniquets, and sponges can limit blood loss and tissue deterioration. However, they have limits in controlling internal bleeding and compressing vital organs including the brain and liver, also they may prolong healing, cause secondary injury and discomfort in the long term [80]. With the developments in nanotechnology, modern hemostatic materials have great potential to control exterior and internal bleeding. Surgicel, QuikClot, Celox®s, MPH, and Singclean's Quickclean and Surgiclean are examples of commonly used products. Surgicel is commonly used in surgeries, however, it has safety issues, including delayed absorption and granuloma formation. QuikClot, a zeolite-based material, stops bleeding by rapidly absorbing water, however also produces heat as a consequence of exothermic reaction. The second-generation QuikClot ACS addresses these difficulties and is more effective, especially on irregular wounds.

Celox®s, a chitosan-based substance, is notably beneficial for patients with coagulation issues and improves wound contact due to its increased surface area [75], [81].

With the improvements in nanotechnology, the properties of materials at the nanoscale can be tailored to provide advantages such as increased diffusivity, higher solubility, ease of penetrating physiological barriers, larger surface area, and controlled drug release [75]. Hemostatic materials can activate platelets by mimicking platelet-activating substances, so assisting in hemorrhage control. For example, collagen mimetic peptides derived from nanofibers can stimulate platelets [82]. Surface roughness and other nanomaterial characteristics influence platelet activation. In secondary hemostasis, negatively charged nanoparticles can activate factor XII, triggering the intrinsic pathway, whereas cationic nanomaterials can activate tissue factors, initiating the extrinsic pathway [83], [84]. Mostly powders, nanoparticles, patches, hydrogels, sponge, and cryogels has been investigated for enhanced hemostatic purposes [85].

1.4.2.1. Patch type

Gauze is one of the commonly used hemostatic material. It has been changed and enhanced several times to meet the demands of increasing emergency and clinical use through many designs, such as dressings, biofilms, bioadhesives, nanofibers, and nanosheets which can be classified under patch type materials [86], [87]. Patch hemostatic materials are sheet-like materials that adhere to bleeding sites, cover and prevent from external harm. Their basic mechanism is to create a physical barrier by sealing over and providing pressure to the wound. Then, these patches use internal pore structures to quickly absorb blood and water, providing platelet aggregation. They are simple to use, provide rapid wound coverage, and are especially useful for surface wounds that require compression to control bleeding [87].

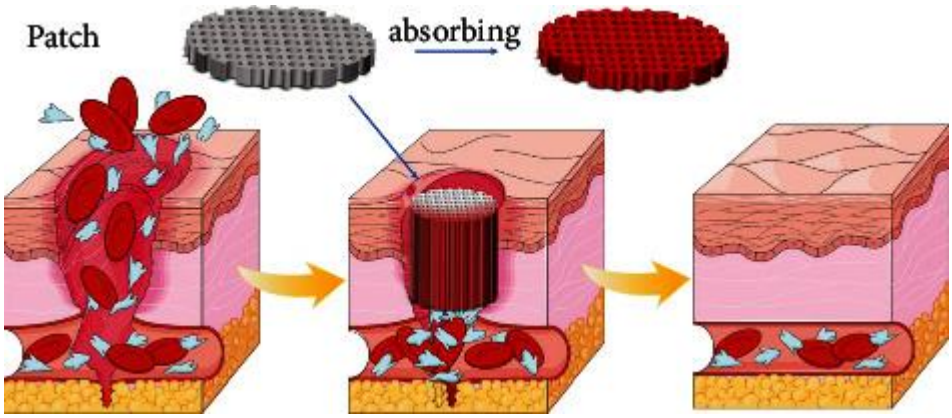


Figure 1.8 Schematic illustration of hemostatic patches [87]**.**

First, to improve the hemostatic efficiency of gauze, biomimetic mineralization of its surface can be achieved. Shi et al. showed that the gauze mineralized by thrombin has the potential to stop bleeding more effectively than the gauze only [88]. Besides the modification of gauze, functional dressings can be produced using natural or synthetic polymers and composite materials. Pandey et al. produced an electrospun nanofibrous patch of polyvinyl alcohol, gelatin, and PLGA enriched by thrombin and vancomycin to control excessive bleeding and antibacterial activity which showed rapid hemostasis and wound healing in a rat model [89]. Xuan et al. developed a two layered flexible nanosheet composed of modified gelatin with calcium ions and mechanically strengthened by a polycaprolactone layer which showed hemostatic and antibacterial properties on skin and liver damage in a mouse model [90]. Moreover, the patches can also be used for drug delivery purposes. In a study, bioadhesive gelatin methacrylate and polyurethane loaded with L-arginine patches were produced and it reduced liver bleeding in rat model while stimulating wound healing by releasing L-arginine [91]. Hemostatic patches can be customized by enhancing water content, morphology, and size. A notable study developed patches with microneedles coated with dodecyl-modified chitosan, which helps catch red blood cells and promotes coagulation. These microneedles physically attach to tissues and are effective for drug delivery. However, the microneedles must be pressed to reach tissues, which poses a risk for applying to fragile organs [92]. Even though patch type hemostatic materials are easy to apply, they are limited by wet adhesion and are likely to fuse with the blood clot which may induce secondary bleeding and discomfort to the patient [93].

1.4.2.2. Powder type

Hemostatic powders, which include nanoparticles, act by absorbing blood, concentrating it, and allowing the powder to adhere closely to damaged tissue in deep and irregular wounds. Positively charged groups in the powder, as in chitosan, interact electrostatically with negatively charged red blood cells which result in localized clot formation and rapid hemostasis [85], [87].

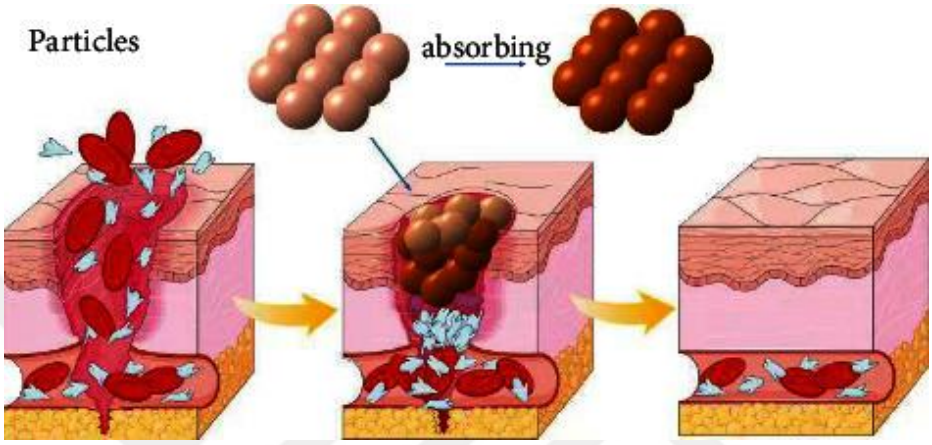


Figure 1.9 Schematic illustration of hemostatic powders [87].

The micro and nano morphology of the powder materials can be customized in different shapes like Janus structure and layer-by-layer assembly [85]. Liu et al. produced polydopamine-coated chitosan/calcium pyrophosphate microparticles with a hierarchical porous structure like a flower, and it exhibited hemostasis by the induction of hemocytes and platelet aggregation while providing biocompatibility with no adverse exothermic reaction [94]. Composite microparticles with multilayer structures can be also utilized. Liu et al. produced microparticles from starch and plant polyphenols for the treatment of bone defect while coating the microporous core structure with quaternized starch and tannic acid through layer-by-layer assembly for intractable bleeding and it showed favorable hemostasis with high biodegradability and bone repair in a mouse model [95]. Peng et al. produced gelatinizable and adhesive polyethyleneimine/polyacrylic acid/quaternized chitosan powder which can absorb blood and form a hydrogel *in situ* for irregularly shaped and noncompressible wounds [96]. Despite the fact that powder is convenient for deep and irregular wounds, it is difficult to provide hemostasis for large wounds and massive bleeding due to low adhesion and being prone to be removed away by blood flow. Further, it has a risk of entering blood circulation which may increase the risk of embolism [87], [97].

1.4.2.3 Hydrogel type

Hydrogels, which have a three-dimensional network mimicking the ECM structure of tissues, are often used in biomedicine. Hydrogels adhere to tissues by physical or chemical interactions to maintain hemostasis. Hydrogels do not typically need to be removed after bleeding has stopped, and with good design, they can aid in wound healing [98]. Several bio-adhesive techniques have been investigated, such as mussel-inspired adhesion, Schiff base chemistry, and NHS ester bonding. Studies have shown that hydrogels may prevent bleeding in organs such as the liver, kidney, and skin [99]. Luo et al. produced two different injectable hydrogel which were self-crosslinking gelatin (Sc-G) and hyaluronic acid/gelatin (HA/G) hydrogels. They were in situ gelatinized and result showed that they HA/G had better hemostatic activity with less gelling time and it bursting strength exceeded fibrin glue [100]. However, most hydrogels are insufficient for excessive bleeding, such as arteriovenous and cardiac [85]. So, bioadhesion, shape adaptation of the hydrogels can be enhanced using photocuring. Hong et al. produced photoreactive adhesive hydrogel from methacrylated gelatin and modified hyaluronic acid which is injected and crosslinked by UV (ultraviolet) to form hydrogel and adhere to the wet tissues through cohesion and aldehyde groups generated after crosslinking that the hydrogel was able to stop high pressure bleeding in a pig artery [101]. Even though hydrogels are convenient for post- hemostasis management and wound healing for being able to mimic ECM structure, wet adhesion ability remains an issue to be improved [85].

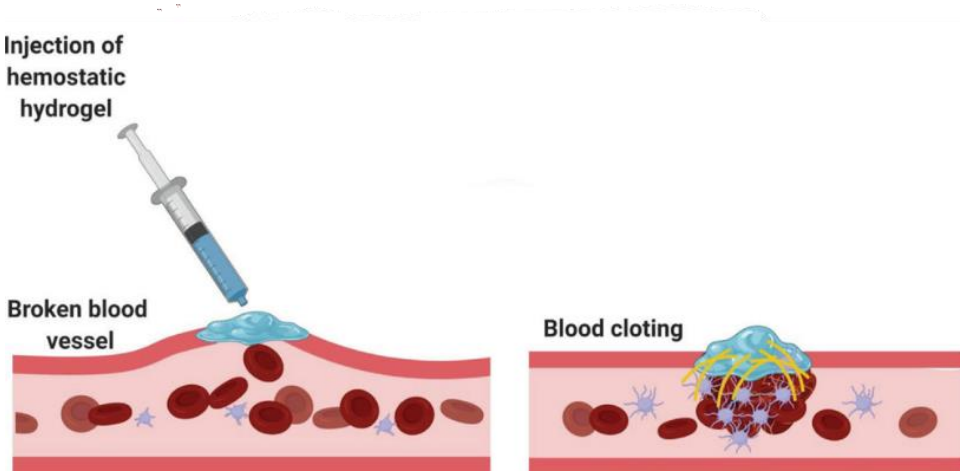


Figure 1.10 Schematic illustration of hemostatic hydrogel injection in a bleeding site [102].

1.4.2.4 Sponge type

Sponge represents a prevalent form of hemostatic materials having high porosity, large surface area, and high swelling capacity which absorbs blood and concentrates procoagulant factors resulting in excellent hemostatic performance [103]. As it absorbs blood and water owing to its porous structure, it fits in the wound and prevents the bleeding by physically sealing and initiating coagulation even in large noncompressible wounds [87].

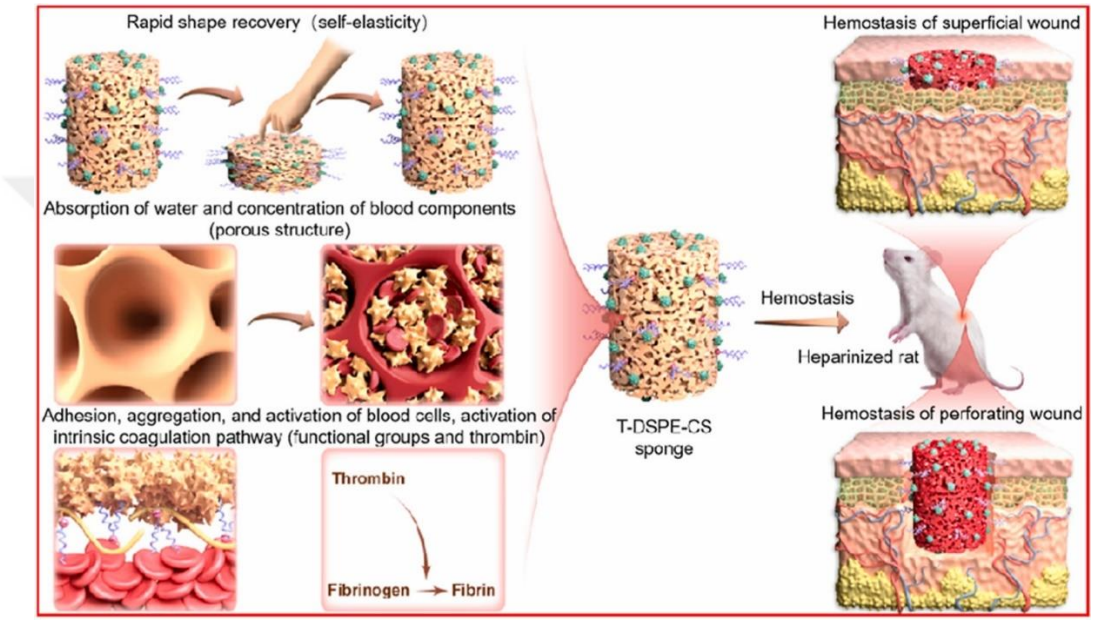


Figure 1.11 Application of a sponge material for superficial and perforating wounds to prevent hemorrhage [104].

Several fabrication methods for sponge materials exists such as foaming, leaching, three-dimensional (3D) printing, and freeze-drying combined with several surface and chemical modifications [85]. Du et al. fabricated chitosan-based sponge via freeze-drying, chemically modified and loaded thrombin to enhance its hemostatic activity. The sponge exhibited better hemostatic efficiency than commercial gauze and gelatin sponge in a hemorrhagic rat tail [104]. Du et al. developed micro channeled alkylated chitosan sponge through combining 3D printed microfiber leaching and freeze-drying to customize pore structure, and superficial active modification to increase antibacterial and hemostatic efficiency which showed better coagulant activity compared to commercial blood stoppers [105]. Cryogels are another type of sponge materials, yet they have superior characteristics due to fabrication technique which provides large pores, interconnectivity among the pores, flexibility, and shape memory. Guo et al. produced shape recovery

cryogels made of carbon nanotube and quaternized chitosan functionalized by glycidyl methacrylate for hemostasis and wound healing. It performed fast and high blood swelling capacity, improved mechanical strength and enhanced platelet adhesion and activation compared to gauze and gelatin sponge in noncompressible hemorrhage application in rabbit liver damage [106]. Also, Yao et al. fabricated a shape memory cryogel composed of quaternized chitosan and mesoporous bioactive glass to control excessive bleeding and promote wound healing. The results showed that the cryogels having highly porous structure exhibiting compression strength maintaining the shape after 10 cycles. It also showed good hemostatic effect compared to gelatin sponge, antibacterial property against gram positive and negative bacteria with accelerated wound healing [107].

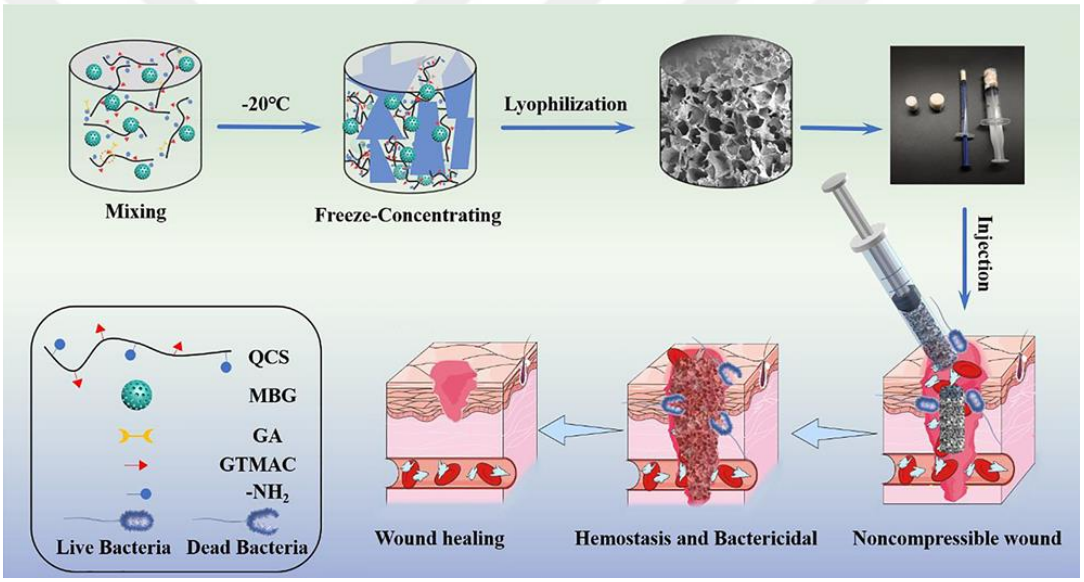


Figure 1.12 Schematic representation of preparation and application as a cryogel for noncompressible wound [107].

1.5 Cryogels: Fabrication and Properties

Gels are polymer networks swollen by a fluid, while hydrogels have been designed from a hydrophilic polymer network with water as the swelling agent [108]. Since Wichterle and Lim developed poly (2-hydroxyethyl methacrylate) hydrogels for contact lenses in 1960, hydrogels have been used in various applications, including superabsorbent polymers, sensors, and soft robots [109]. However, first-generation

hydrogels have low mechanical strength because of chemically cross-linked network structure that lacks energy dissipation. To improve the mechanical performance of hydrogels, new design strategies have been developed [110]. One such technique is conducted by Gong et al. pioneering work on double-network hydrogels, that combine brittle and ductile networks. The brittle network releases energy by breaking under stress, whereas the ductile network preserves the material's structural integrity [111]. Another novel advancement in the field is cryogelation, a process used to fabricate highly porous, mechanically resilient hydrogels known as cryogels. Though cryogels were discovered over 70 years ago, they have gained significant attention in the recent two decades, as demonstrated by an increase in research papers [110], [112].

Cryogels are fabricated using cryogelation, which results in either chemically or physically crosslinked networks depending on the initial ingredients [113]. The reaction mixture composed of monomers or polymeric precursors dissolved in a solvent [108]. The freeze-thawing technique requires the free-radical copolymerization of monomers and cross-linkers in solvents, such as water, dioxane, or dimethyl sulfoxide (DMSO) [114]. When the solvent is cooled below its freezing point, ice crystals form and function the development of pores, allowing the polymer network to assemble around them and form the interconnected macroporous structure of cryogels after being thawed [115]. Chemically crosslinked cryogels are formed by polymerizing and crosslinking around ice crystals, whereas physically crosslinked cryogels rely on chain entanglements or crystalline regions. To achieve a stable structure in physically crosslinked systems, several freeze-thawing cycles may be required [108], [116].

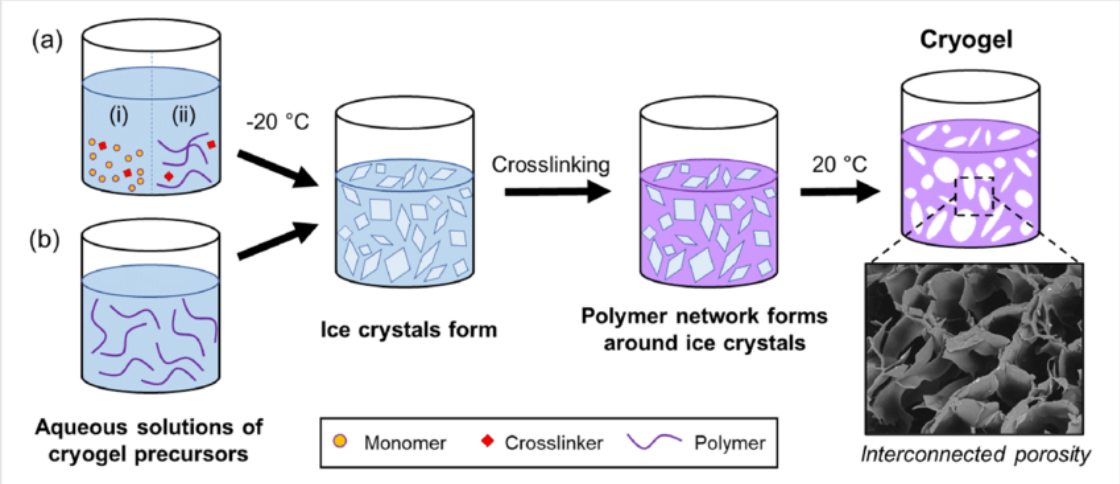


Figure 1.13 Schematic representation of the cryogel's fabrication [108].

Crosslinking agents are used in the production of porous scaffolds such as hydrogels and cryogels. These are mainly APS/TEMED (Ammonium persulfate/N,N,N',N'tetramethylethylenediamine), EDC/NHS (Ethylene dichloride/N-hydroxysulfosuccinimide), genipin and glutaraldehyde [117], [118]. Glutaraldehyde is an agent widely used in cryogel production and is used to form a physically crosslinked network structure by reacting with amine groups. It forms a physically crosslinked network structure in polymers [115]. The use of glutaraldehyde at high concentrations can cause toxic effects in biomaterial production. However, some studies have observed that the use of glutaraldehyde at low concentrations does not cause cytotoxicity. In a study, the toxic effect of glutaraldehyde used for chitosan/gelatin cryogels was investigated. As a result, chitosan/gelatin scaffolds showed increased cellular metabolic activity in fibroblast cells over time, and it was observed that glutaraldehyde had no effect on cell proliferation and growth. In addition, since the glutaraldehyde concentration used in the study was between 1% and 0.3% (w / v), it was stated that cytotoxicity was not observed at low concentrations [119]. In another study, synthesized chitosan-gelatin cryogels with glutaraldehyde did not show cytotoxicity although they used a high glutaraldehyde concentration of 6% (w / v) in their study [120].

These macroporous hydrogels, fabricated using cryogelation, have distinct features such as high porosity with developed macro pores that are homogeneously interconnected in a three-dimensional network structure, elasticity, and efficient diffusion, making them appropriate for a variety of biomedical applications [121]. Cryogels have several advantages over regular hydrogels, including increased mechanical strength, ease of handling, storage, and sterilizing [113]. It also has been suggested that the hemostatic effect of cryogels was superior than hydrogels due to their unique pore structure and swelling ratio with the same composition [85].

1.5 Chitosan and Gelatin Cryogels in Hemostatic Applications

The interconnected macroporous structure of the cryogels can create a hemostatic effect by providing a surface area for blood cells to adhere and initiate coagulation cascade through contact activation and aggregation of the platelets [106], [122] .In addition, cryogels swell blood, which ensures their high blood absorption capacity and creates a physical barrier by closing the wound [123].

Chitosan, a natural polymer, is a deacetylated form of chitin and is used in hemostatic materials as a functional biopolymer.[124] The main known features of chitosan are its non-toxicity, high biocompatibility and antimicrobial properties. In addition, it increases platelet aggregation and accelerates hemostasis by electrostatic interactions between the anionic groups of red blood cells surface such as phosphatidylserine, neuraminic acid residues, and cationic groups in its structure [125], [126]. Similarly, it provides platelet activation and aggregation by increasing the formation of the glycoprotein complex and initiates the coagulation pathway with its swelling feature when in contact with blood. [41] The antibacterial and hemostatic characteristics of the chitosan is influenced by the cationic nature of it which is why the derivatives with the addition of quaternary ammonium groups such as L-cysteine to form thiol chitosan, and mono(2-methacryloyloxyethyl) ester of phosphoric acid to produce phosphorylated chitosan [127], [128].

Gelatin is another natural polymer synthesized by hydrolysis of collagen with known biocompatibility and hemostatic effect [75]. Since gelatin has high liquid absorption properties, it can act as a physical plug when applied to damaged tissue and has a hemostatic effect by concentrating red blood cells and increasing platelet accumulation [129]. Moreover, gelatin can entrap the negatively charged erythrocytes into the blood clot to constitute fibrin mesh via cationic groups in its backbone structure [130]. There are several gelatin-based products present at the market such as Gelfoam® and Surgifoam®, yet chemical modifications and composites of the gelatin have been used to enhance its hemostatic activity [131]. A study performed modification of methacrylic anhydride in gelatin to increase adhesion of the gelatin for hemostasis and wound sealing while another study produced composite of alginate/gelatin sponge with nanofibers including curcumin for rapid hemostasis and tumor formation [132].

Chitosan/gelatin cryogels are a suitable model for hemostatic applications due to their properties. Chitosan-gelatin composite cryogels show better absorption capacity and platelet aggregation than chitosan and gelatin cryogels alone without causing any cytotoxicity [133]. However, chitosan/gelatin-based biomaterials are still limited and the hemostatic activity can be increased blending with bioactive substances [134] such as oxidized cellulose, silicate nanoparticles, calcium [78], [79].

1.6 Usage of Plant Extracts for Hemostasis

Plants have long been used for their hemostatic properties to control bleeding and aid in wound healing [135]. Their bioactive compounds, which interact with the body's coagulation pathways, have become a focus of modern research due to their potential to offer natural alternatives to synthetic hemostatic agents [136]. Several studies have explored the hemostatic effects of plant species. For example, *in vitro* research on the extracts of *Urtica urens, Verbascum fruticulosum, Parietaria judica, Lupinus pilosus, Satureja thymbra, Thymbra spicata, Teucrium creticum,, Paronychia argentea, and Ruta chalepensis* in ethanol demonstrated both anticoagulant and coagulant activities depending on the species [137]. Additionally, extracts from *Satureja thymbra*, *Verbascum fruticulosum*, and *Thymbra spicata* were examined for their hemostatic effects, with flavonoids identified as key bioactive components [138]. Phytochemicals such as flavonoids, phenols, and tannins are found in many plants and play critical roles in promoting hemostasis. Flavonoids, present in species like *Verbascum fruticulosum*, have been shown to improve platelet function and influence clotting times [139]. Phenolic compounds, including terpenoids, affect the blood clotting process by activating platelet factor XII and regulating thrombin–fibrinogen interactions [140]. Tannins also contribute to the coagulation cascade and further enhance hemostatic effect by shortening prothrombin time (PT) and activated partial thromboplastin time (aPTT) [141]. These bioactive compounds act synergistically, offering potent hemostatic properties based on the specific plant species and extraction methods [139].

When some studies in the literature are examined, the usage of natural and synthetic polymers together with plant-derived substances creates an innovative therapeutic approach, allowing more effective results to be obtained. Thus, alternative treatment approaches and the use of natural plant extracts have gained importance in recent years [142].

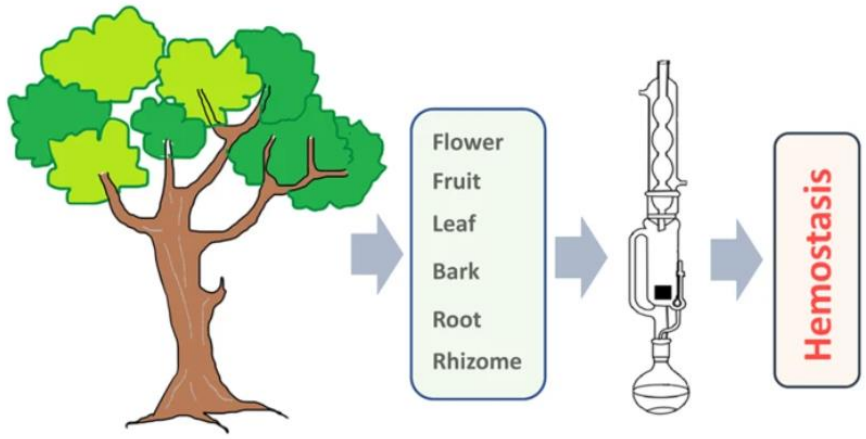


Figure 1.14 Plants are extracted through various techniques like maceration, decoction, soxhlation, and microwave extraction to yield active components to be used in hemostasis [19].

1.6.1 *Verbascum Thapsus*

In this study, we investigated the *Verbascum Thapsus* extract for its hemostatic and antibacterial activity. *V. thapsus*, also known as mullein, is a hairy plant found in Europe, North Africa, and Asia and has been used traditionally for centuries [143]. In the literature, this medicinal plant has analgesic, antihistamine, anti-inflammatory, and antiseptic effects, as well as anti-tumor, antioxidant, and antibacterial effects [144], [145]. Traditionally, the infusion of its flowers in olive oil is very effective in destroying the microbes that cause the disease and has been used in regional treatments such as earache, gum and mouth ulcers while its boiled roots have been used as a sedative for migraines and headaches, toothaches and cramps. Its dried leaves have been used for irritation of mucous membranes, coughs and asthma. It is also used externally in medicine for burns, ulcers and hemorrhoids [145], [146].

Figure 1.15 Natural appearance of *Verbascum thapsus* (Kayseri, Türkiye).

There are several pharmacological studies about *Verbascum thapsus* and its extract in the literature, however, there is no study about a hemostatic biomaterial containing *V. thapsus*. To control external bleeding, besides physical sealants and absorbable materials, bioactive hemostatic materials should be developed from polymers to be more efficient [142]. Especially essential plant oils and extracts are most preferable as bioactive agents due to their unique properties [147]. In this study, we combined the three mechanisms of hemostatic materials in one to achieve superior hemostatic activity and engineered a hemostatic biomaterial produced from a chitosan/gelatin cryogel containing *V. thapsus* plant extract. The obtained hemostatic biomaterial was characterized morphologically, chemically, physically and biologically. Further, *in vitro* hemostatic activity of the cryogels were evaluated to be suitable for clinical applications.

Chapter 2

Experimental Section

2.1 Materials

Firstly, *V. thapsus* plant was obtained from (July 2023) Kayseri/ Türkiye. Gelatin (type B, 225g bloom, bovine skin), Chitosan (Mw: low 50-190 kDa, %75-85 deacetylated), Glutaraldehyde (25%), acetic acid, Phosphate buffered saline (PBS), Trypsin-EDTA (0.25%) were purchased from Sigma. Dulbecco's Modified Eagle Medium (DMEM, High Glucose) and Penicillin-Streptomycin, Fetal Bovine Serum (FBS) were obtained from Serox. Methylthiazolyldiphenyl-tetrazolium bromide (MTT), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), Dimethyl sulfoxide (DMSO), Calcium Chloride, Sodium Citrate, were purchased from Sigma Aldrich (USA). The human dermal fibroblast (HDFa, PCS-201-010), *Escherichia coli* (ATCC BAA-1025) and *Staphylococcus aureus* (ATCC 6538) were purchased from ATCC. LB Broth, LB Agar, ethanol, and methanol were purchased from Merck. The commercial blood stopper was obtained from a local medical market. The sheep blood used in blood tests was collected from slaughtered sheep at a local slaughterhouse, Erkem Meat Integrated Facility (Kayseri/ Turkiye).

2.2 Methods

2.2.1 Extraction and Characterization of *V. thapsus*

Firstly, 25 g of dried *V. thapsus* is extracted in methanol by the Soxhlet technique at 60°C for 8 h. After extraction, the methanol was removed from the extract using a rotary evaporator (Buchi, Rotavapor R-300), and the final extract was stored at 4°C for further use. The chemical composition of *V. thapsus* extract was investigated by gas chromatography-mass spectrometry (GC-MS) analysis (GCMS-QP2010 Ultra, Shimadzu). The analysis was performed using helium as a carrier gas at a constant flow rate of 2 mL/min inside the capillary column (Rtx-5MS, 30 m x 0.25 mm x 0.25 µm, Restek 12641). The temperature increased from 40 °C to 280 °C at a rate of 15 °C/min. Afterward, *V. thapsus* extract dissolved in methanol was injected in the split-less mode at 280°C.

2.2.2 Fabrication and Optimization of Chitosan/Gelatin Cryogels

Chitosan/gelatin cryogels were fabricated through the crosslinking of gelatin and chitosan with glutaraldehyde. A total of 4% (w/v) polymer concentration of chitosan: gelatin was dissolved in 4 mL acetic acid (1%, v/v) and 1 mL of glutaraldehyde (0.025%, v/v) was then added to the solution. The total 5 mL polymeric solution was immediately poured into 2.5 mL syringes and incubated at -18 °C for 24 hours. After incubation, the chitosan/gelatin cryogels were freeze-dried (Labconco, FreeZone) overnight and washed with distilled water.

Considering the glutaraldehyde concentrations and chitosan/gelatin ratios in the literature, 25 different chitosan/gelatin cryogels were produced (Table 3.2.1). These 25 cryogels were evaluated based on morphological observations, physical analysis, and shape deformations. Finally, C9, C10, C14, C15, C19, and C20 cryogels were chosen for further morphological and physical analysis which were SEM and swelling capacity test, respectively.

2.2.3 Morphological and Physical Characterization of Chitosan/Gelatin Cryogels

The selected cryogels' macroporous structure and pore sizes were investigated by Scanning Electron Microscopy (SEM, GeminiSEM 300, Zeiss) at magnifications 50X, 30X, and 20X [117]. The ImageJ software (National Institutes of Health, Bethesda, MD) was used to calculate the average pore sizes of the cryogels from SEM images. The swelling capacity of these cryogels was investigated in PBS at 37 °C. Before and after PBS absorption, cryogels were weighed at 5-minute intervals for 60 minutes, and swelling capacities were calculated using the following equation;

(%) Swelling Capacity = [(wet weight – dry weight)/dry weight] x 100 Equation 1 [148]

2.2.4 Obtaining and Characterizations of the Cryogels Containing *V. thapsus*

During the extract impregnation step, dried *V. thapsus* extract was diluted in 70% ethanol at concentrations of 1%, 5%, 10%, and 20% (g/mL) and absorbed by the chitosan/gelatin cryogel. The cryogels were dried after being impregnated with the extract.

2.2.4.1 Morphological Characterization

The morphology and macroporous structure of the cryogels containing *V. thapsus* extract were investigated by SEM at magnifications of 50X, 30X, and 15X.

2.2.4.2 Fourier Transform Infrared (FT-IR) Analysis

Chemical structures of the cryogels containing *V. thapsus* extract were characterized using FT-IR Spectrometer (Thermo Scientific, Nicolet 6700), in the range of 400-4.000 cm^{-1} [149].

2.2.4.3 Swelling Capacity

The swelling capacity of the cryogels containing *V. thapsus* extract was investigated in PBS and citrated blood at 37 °C. Before and after being swelled, cryogels were weighed at 5-minute intervals for 60 minutes, and swelling capacities were calculated using the equation 1.

2.2.4.4 Mechanical Analysis

The compression test was performed to investigate the mechanical strength of the cryogels [117]. First, the cryogels were prepared in a cylindrical shape with a height of 6 mm and a diameter of 12 mm. Then, they were swollen in PBS for 15 minutes. Compression was applied uniaxially to swollen cryogels at 50N per load with a speed of 2 mm/min. Stress-strain curve was plotted by strain (%) on the *X-axis* and stress (KPa) on the *Y-axis.* The compression modulus was then calculated from the slope of the graph at 60-70% strain region [150], [151].

2.2.4.5 Antibacterial Activity

The antibacterial activity of the cryogels was investigated by calculating bactericidal ratios against *E. coli* (ATCC 25922, Gram-negative) and *S. aureus* (ATCC 6538, Gram-positive) bacteria. 50 µL of bacterial suspension diluted with sterile PBS ($1x10^6$ ml^{-1} cell) was added to the cryogels and incubated at 37 °C for 2 hours. For the control group, the bacterial suspension was placed in an empty test tube. The samples were suspended in 1 mL of sterile PBS and the cryogels were removed. Then, 4 mL of Luria-Broth (LB) media was added and incubated for 16 hours at 37 °C. A 200 µL sample was obtained from the bacterial suspension culture and transferred to a 96-well plate. The OD value was measured using a microplate reader (Varioskan, Thermo Scientific,) at 600 nm. The method was repeated three times for each group. As a result, the bactericidal ratio was calculated as a percentage using the equation below.

$$\% \text{ Bactericidal ratio} = (OD_{control}-OD_{sample})/ OD_{control} \quad \text{Equation 2 [107]}$$

2.2.4.6 *In vitro* Cell Viability

The cell viability of cryogels was investigated using a human fibroblast cell line (HDFa, PCS-201-010, ATCC) using an MTT assay (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma) by direct contact method [152]. First, fibroblast cells were cultured on cell culture plates using DMEM-High Glucose medium supplemented with 10% FBS and 1% penicillin/streptomycin in an incubator at 37 °C and 5% CO_2 [153]. All cell culture studies were carried out in a laminar flow hood following the sterile aseptic techniques. The medium was refreshed every two days, and cells were passaged when they reached 90% confluence. The cryogels were prepared in accordance with ISO 10993-5:2009(E). The cryogels were sterilized for 1 hour with UV irradiation before being placed in 96-well plates. Each well was seeded with 10^4 cells in 200 µl DMEM. The cryogels were then added on the wells and incubated for 24, 48, 72 hours at 37 °C with 5% CO_2. After incubation, cryogel samples were removed and 20 µl of MTT reagent was added to each well. The well plate was incubated for 2 hours and centrifuged at 1800 rpm for 10 minutes. The supernatants were removed from each well and 100 µl of DMSO was added to dissolve formazan crystals. Finally, the absorbance of each well was measured at 570 nm using a microplate reader (Varioskan, Thermo Scientific) [154].

2.2.5 Hemostatic Activity of the Cryogels Containing *V. thapsus*

2.2.5.1 Hemolysis Assay

Hemocompatibility of the cryogels was investigated by hemolysis assay. First of all, sheep blood was taken, and 3.8% sodium citrate was immediately added to prevent blood clotting prior to the experiments. The citrated blood was used for further whole-blood test experiments. The cryogels were placed in 5 ml of 0.9% NaCl physiological salt solution. The negative control was a solution containing 2% blood and 98% physiological salt, while the positive control was a solution containing 2% blood and 98% distilled water. 100 µL of citrated blood was added to the cryogels and incubated at 37 °C for 30 minutes. After centrifugation at 1000 G for 10 minutes, 100 µL of supernatants were transferred to a 96-well plate. The absorbance of each well was recorded at 545 nm, and then the hemolysis rate was calculated based on the equation below.

% Hemolysis rate = $[(A_s-A_n)/(A_p-A_n)] \times 100$ Equation 3 [155]

where A_s is the absorbance of the tested cryogel samples, A_n and A_p are the negative control group and positive control group, respectively.

2.2.5.2 *In vitro* Whole Blood Clotting

The hemostatic effect of the cryogels was investigated by whole blood clotting assay. First, the cryogels prepared in a cylindrical shape with 1 cm thickness and a commercial blood stopper were placed in glass test tubes. The 200 µL of citrated blood was added to the samples, then 20 µL of $CaCl_2$ (0.2 M) was quickly added and incubated at 37 °C for 5 min. The 10 ml of distilled water was carefully added to glass tubes to hemolyze hemoglobin that was not adsorbed or engaged in clot formation on the samples. The absorbance of the final blood-sample solution was measured at 540 nm. The absorbance of citrated whole blood in deionized water was used as a reference (negative control), and the blood clotting index (BCI) of the samples was calculated using the equation below.

BCI = (Absorbance of the sample/Reference absorbance) x 100 Equation 4 [156]

2.2.5.3 *In vitro* Whole Blood Clotting Time

The cryogels were prepared as 0.5 x 0.5 x 2 mm and placed on 24-well plate. The citrated blood was mixed with $CaCl_2$ (25 mM, 9:1 volumetric ratio) and added to the cryogels and CBS (Commercial blood stopper). Following that, 200 µL of blood was pipetted onto each sample and an empty well was added with only blood as a control group. At predetermined time intervals, each well was washed with a saline solution (NaCl 0.9% w/v) until the solution became clear, leaving the clot in the well plate. The time lapse for 1, 3, 5, 10, 15, 20, 25 and 30 minutes was figured. Also, the clotting time required to form a persistent clot on the surface of the cryogel samples was recorded.

2.2.5.4 Platelet Adhesion

The adherence of blood platelet cells on the surface of the cryogels was investigated under the fluorescence microscope and SEM. For this, 200 μL of citrated blood was added to the sterile cryogel sample and incubated at 37ºC for 5 minutes. After washing the cryogel with PBS to remove non-adherent platelets, it was incubated with glutaraldehyde (2.5%, v/v) for 2 hours at 4ºC. After incubation, cryogels were dried, and DAPI staining was used to determine the cell nuclei of the platelets, and samples were examined under the SEM and fluorescence microscopes. The SEM images were colorized by using Mountains10 software.

Chapter 3

Results and Discussion

3.1 Characterization of *V. thapsus*

V. thapsus is known as great mullein or common mullein, a biennial plant species belonging to the *Scrophulariaceae* family [157]. The phytochemical profile of the obtained methanolic extract of *V. thapsus* was investigated by GC-MS analysis, and the results are represented in Figure 3.1. The *V. thapsus* extract contains hexanal (7.17%), benzoic acid (methyl 2-methoxybenzoate, 5.34%), hexanal dimethyl acetal (2.91%), benzaldehyde 2,5-bis[(trimethylsilyl)oxy]- (4.50%), Bicyclo (2.2.1) heptan-2-one, 1,7,7-trimethyl-, (1S)- (4.77%), 2-undecenal (4.50%), trans-4,5-epoxy-(E)-2-decenal (1.18%), hexadecanoic acid methyl ester (5.87%), hexadecanoic acid (palmitic acid, 8.81%), 11,14-eicosadienoic acid methyl ester (2.66%), 9,12-octadecadienoyl chloride (linoleoyl chloride, 8.09%), (Z)6(Z)9-pentadecadien-1-ol (24.66%), octadecanoic acid (stearic acid, 5.31%), and gamma-sitosterol (clionasterol, 14.23%). The obtained phytochemicals from *V. thapsus* extract were compared with data reported in the literature, and it was reported that the benzoic acid is one of the important phenolic components in *Verbascum* species [158]. Hexanal belongs to the fatty aldehydes group, and 18-carbon stearic acid belongs to the fatty acids group in *Verbascum* species [159], [160]. The main saturated or unsaturated fatty acids include lauric acid, tetradecanoic acid, 3-hydroxyundecanoic acid, 2-hydroxytetradecanoic acid, 9-hexadecenoic acid, 7-hexadecenoic acid, hexadecanoic acid, 10-methylenehexadecanoic acid, heptadecanoic acid, 12-octadecadienoic acid, cis-9-octadecadienoic acid, stearic acid, and cis-9,10-methyleneoctadecanoic acid [161]. Additionally, in another study, pentadecadien, hexanal, and hexadecanoic acid were reported as the main phytochemicals found in *V. thapsus* [162], [163]. The hexanal, benzaldehyde, sitosterol, palmitic acid, and stearic acid were confirmed with the

literature, [143], [162], [164], it can be concluded that the *V. thapsus* was successfully extracted.

a)

Peak#	Retention Time	Compound's Name	NIST Library
1	5.144	Hexanal	Caproaldehyde
2	8.748	Benzoic acid, 2-methoxy-, methyl ester	o-Anisic acid
3	12.565	Hexanal Dimethyl Acetal	1,1-Dimethoxyhexane
4	22.508	Benzaldehyde, 2,5-bis[(trimethylsilyl)oxy]-	
5	23.289	Bicyclo [2.2.1] heptan-2-one, 1,7,7-trimethyl-, (1S)-	Camphor
6	30.471	2-Undecenal	
7	35.032	trans-4,5-Epoxy-(E)-2-decenal	4,5-epoxy-(E)-2-decenal
8	72.370	Hexadecanoic acid, methyl ester	Palmitic acid, methyl ester
9	76.048	Hexadecanoic acid	Palmitic acid
10	80.563	11,14-Eicosadienoic acid, methyl ester	
11	80.871	9,12-Octadecadienoyl chloride, (Z, Z)-	
12	84.397	(Z)6, (Z)9-Pentadecadien-1-ol	
13	85.299	Octadecanoic acid	Stearic acid
14	114.033	Gamma-Sitosterol	

b)

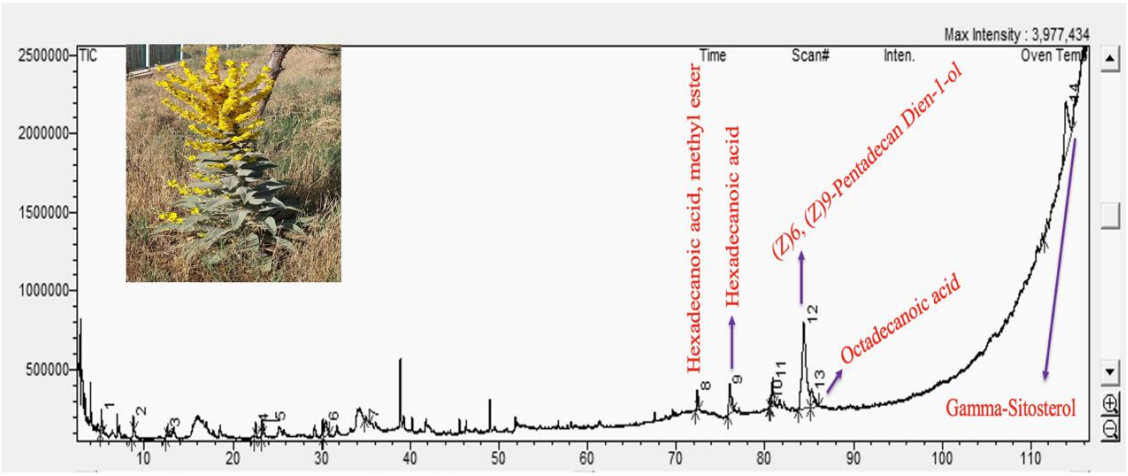


Figure 3.1 a) The phytochemicals and b) GC-MS spectra of *V. thapsus* methanolic extract.

3.2 Optimization, Morphological and Physical Characterizations of Chitosan/Gelatin Cryogels

The 25 different cryogel samples were fabricated by crosslinking with glutaraldehyde while keeping the total polymer concentration constant at 4% (w/v). Firstly, we optimized the chitosan/gelatin ratios to 100:0, 80:20, 50:50, 20:80, and 0:100, (w/w) with different glutaraldehyde concentrations of 1, 0.5, 0.1, 0.05, and 0.025% (v/v) as shown in Table 3.1. Glutaraldehyde was used to crosslink the amino groups in gelatin and chitosan, forming a polymer network structure. During cross-linking, the solution was immediately frozen at -20°C, causing the solvent to crystallize and act as a porogen. After thawing, the scaffold structure results as sponge-like and macroporous [165]. After the macro-observation of fabricated cryogels, 19 of them was eliminated due to their insufficiency for cryogel usage as seen in Figure 3.3.a. The C9, C10, C14, C15, C19, and C20 cryogels were selected as optimum cryogel, and then investigated morphologically by SEM.

Table 3.1 Cryogel samples with different ratios of chitosan: gelatin (C: G, w/w) and glutaraldehyde concentration (GLA, v/v).

Samples	C: G (w/w)	GLA (v/v)	Samples	C: G (w/w)	GLA (v/v)	Samples	C: G (w/w)	GLA (v/v)
C1	100:0	1%	C10	20:80	0,025%	C19	20:80	0,05%
C2	100:0	0,5%	C11	50:50	1%	C20	20:80	0,025%
C3	100:0	0,1%	C12	50:50	0,5%	C21	100:0	1%
C4	100:0	0,05%	C13	50:50	0,1%	C22	100:0	0,5%
C5	100:0	0,025%	C14	50:50	0,05%	C23	100:0	0,1%
C6	80:20	1%	C15	50:50	0,025%	C24	100:0	0,05%
C7	80:20	0,5%	C16	20:80	1%	C25	100:0	0,025%
C8	80:20	0,1%	C17	20:80	0,5%			
C9	80:20	0,05%	C18	20:80	0,1%			

Porosity and interconnectivity are essential for an adequate scaffold, regardless of the tissue type. According to SEM results in Figure 3.2, the chitosan: gelatin cryogels with the 80:20, 50:50, and 20:80 ratios of demonstrated different pore structures and homogeneity. Figure 3.3.b represents the pore diameters of the cryogels, with average pore diameters of the C9, C10, C14, C15, C19, and C20 was 478 ± 280 µm, 359 ± 211 µm, 434 ± 307 µm, 225 ± 49 µm, 443 ± 200 µm and 447 ± 237 µm, respectively.

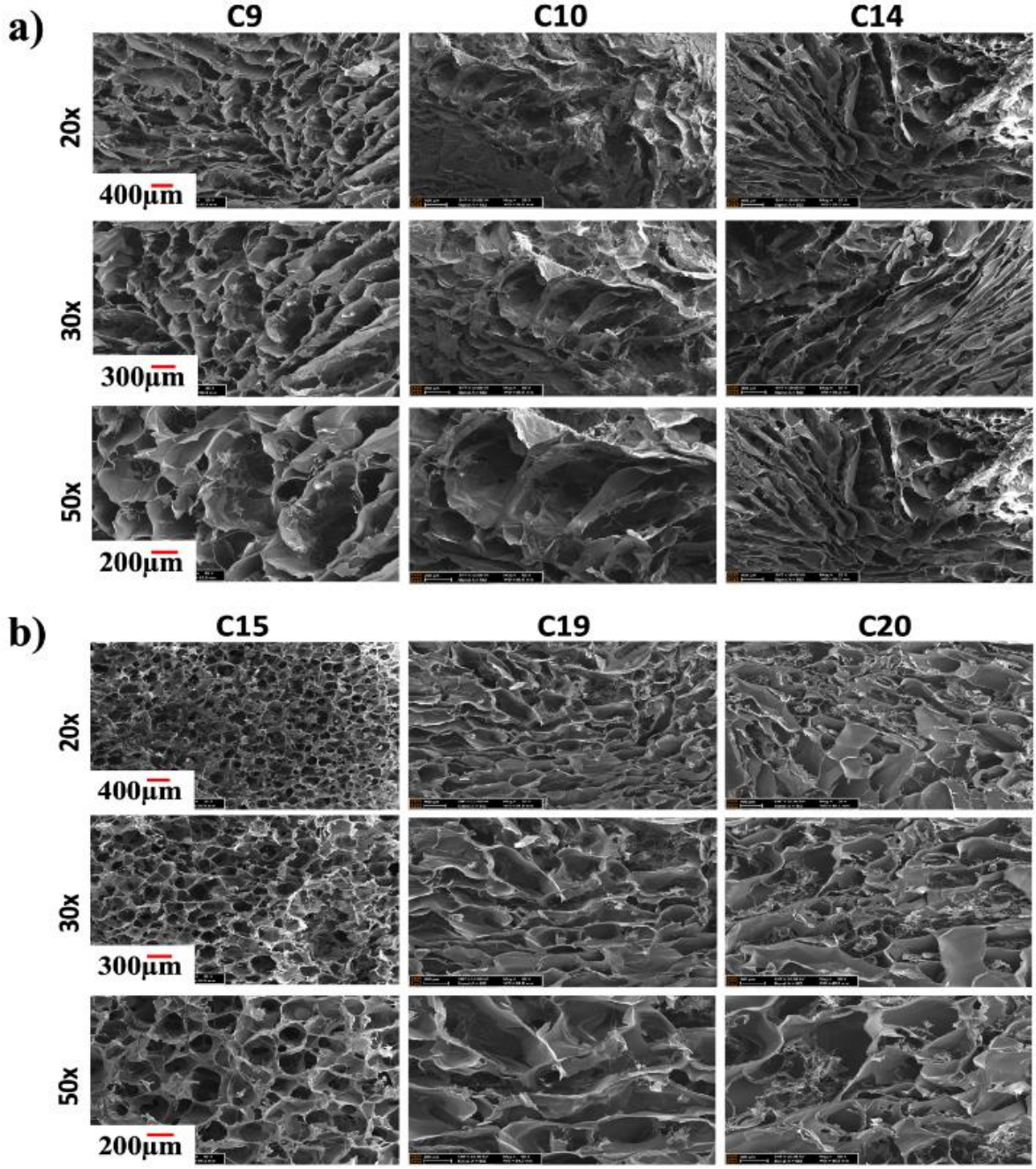


Figure 3.2 SEM images of a) C8, C9, C14, b) C15, C19 and C20 cryogels, with the 20X, 30X, and 50X magnifications.

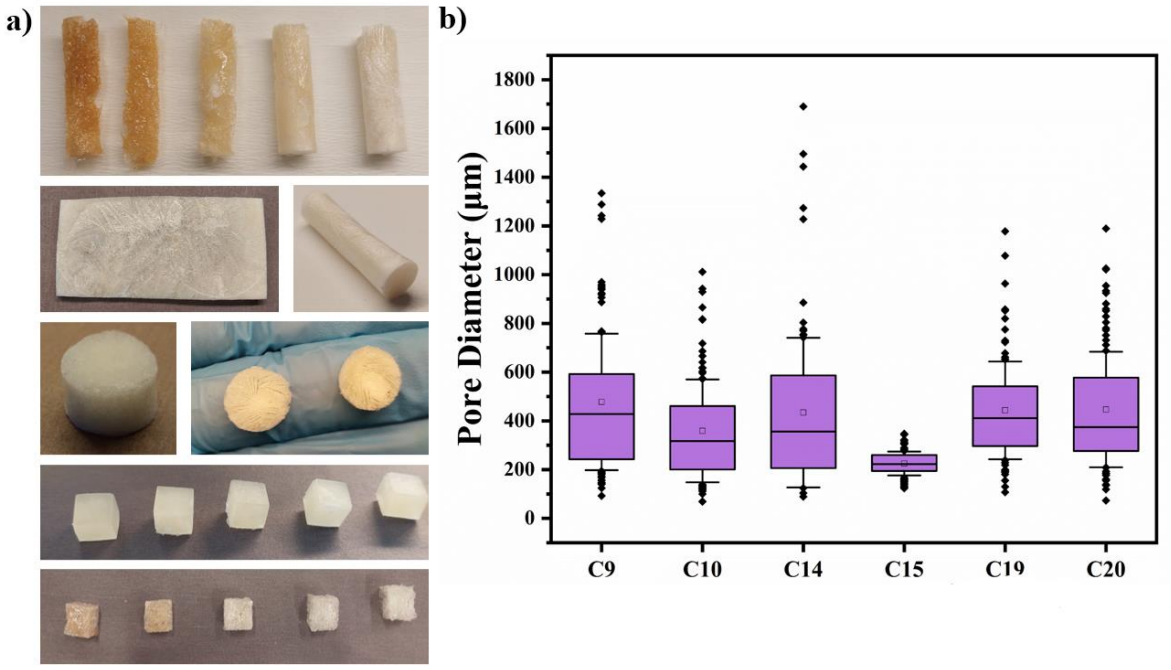


Figure 3.3 a) The fabricated different shaped and sized cryogels, b) The average pore diameters of the cryogels (n= 100)

The pore structures of cryogels can vary depending on factors such as the monomer, cross-linker, solvent, and freezing temperature used in cryogelation [118]. In our results, cryogels with a chitosan: gelatin ratio of 20:80 demonstrated thicker pore walls and irregular pore sizes compared to others. This can be explained by the cryoconcentration phenomenon, which increases pore wall thickness in cryogels as polymer density increases in unfrozen area. Increasing the density of the polymer or solution allows more polymer cross-linking and the formation of thicker pore walls [110]. Moreover, increasing the concentration of the cross-linker agent increases the stiffness of the gel in unfrozen areas, leading to the formation of pores with thicker walls and irregular sizes [115]. Higher crosslinker concentration also influences the reaction kinetics, specifically the competition between the gelation rate and ice crystal formation rate [117]. Therefore, C15 cryogel produced with a chitosan: gelatin ratio of 50:50 and 0.025% (v/v) glutaraldehyde exhibited more homogeneously distributed pore structure with thinner pore walls.

Swelling capacity is an important characteristic of hemostatic materials to be swollen and form a physical barrier preventing blood flow. Thus, the interconnected macro-porous structure of the cryogel is advantageous for capturing blood within the material and concentrating platelets to initiate and accelerate the coagulation process. The swelling ratio of the selected cryogels was tested in PBS for one hour. As shown in Figure

3.4, the C8, C9, C14, C15, C19 and C20 samples had swelling ratios up to 2765%, 2859%, 2140%, 3348%, 2836%, and 2371%, respectively. According to the results, the C15 had a higher swelling capacity than the other samples. It can be explained by the highly homogenous interconnected pore structure keeping water molecules within. Research indicated that cryogels with uniform pore structures perform higher swelling behavior [110], [117]. Similar to our findings, chitosan-based cryogels in the literature showed a swelling ratio of about 3500% [166]. As a result, sample C15 was chosen as the optimum final cryogel due to its more regular and interconnected pore structure and high swelling ratio, and was used in further experiments.

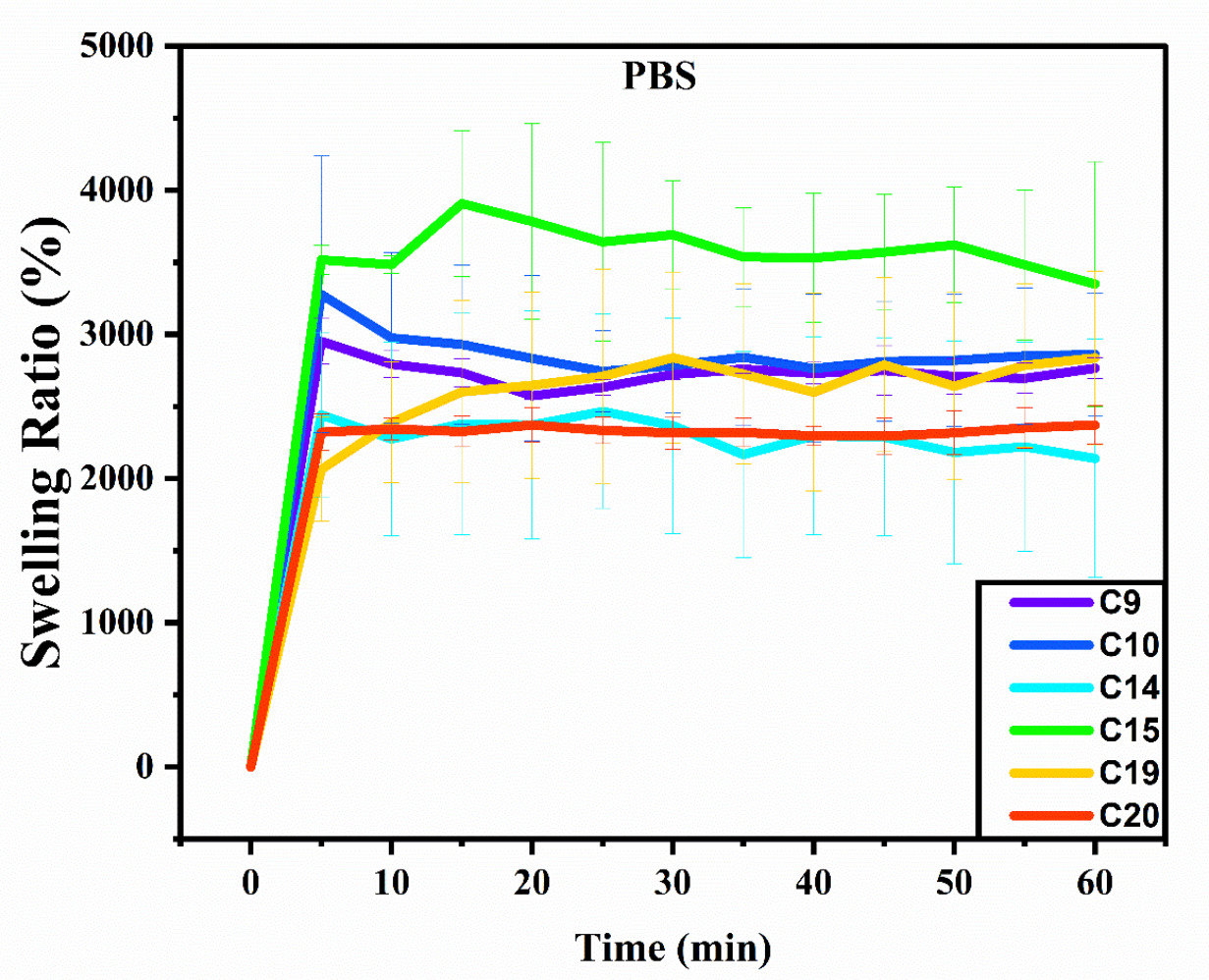
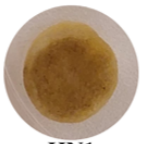


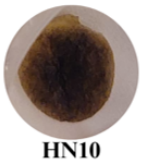
Figure 3.4 Swelling ratio (%) of the cryogels (n=3).

3.3 Characterizations of the Cryogels Containing *V. thapsus*

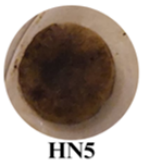
The *V. thapsus* impregnated cryogels were labelled as HN, HN1, HN5, HN10 and HN20, as seen in the Table 3.3.1.

Table 3.2 The nomenclature of final cryogels with and without *V. thapsus* extract.





Cryogels	Chitosan:Gelatin (w/w)	VT concentration (%, g/mL)
HN	50:50	0
HN1	50:50	1
HN5	50:50	5
HN10	50:50	10
HN20	50:50	20



3.3.1 Morphological Characterization

The pore morphologies of *V. thapsus* containing cryogels were investigated using SEM, as shown in Figure 3.5. The pore structure of cryogels had been slightly altered when *V. thapsus* extract was impregnated into them. This can be explained by the usage of 70% ethanol to dilute the extract. Generally, after impregnating the extract, a slight shrinkage is observed in the structure of the cryogel, causing the pore structures to narrow down. However, the pore structures of the obtained cryogels do not pose an obstacle to their application as a hemostatic material.

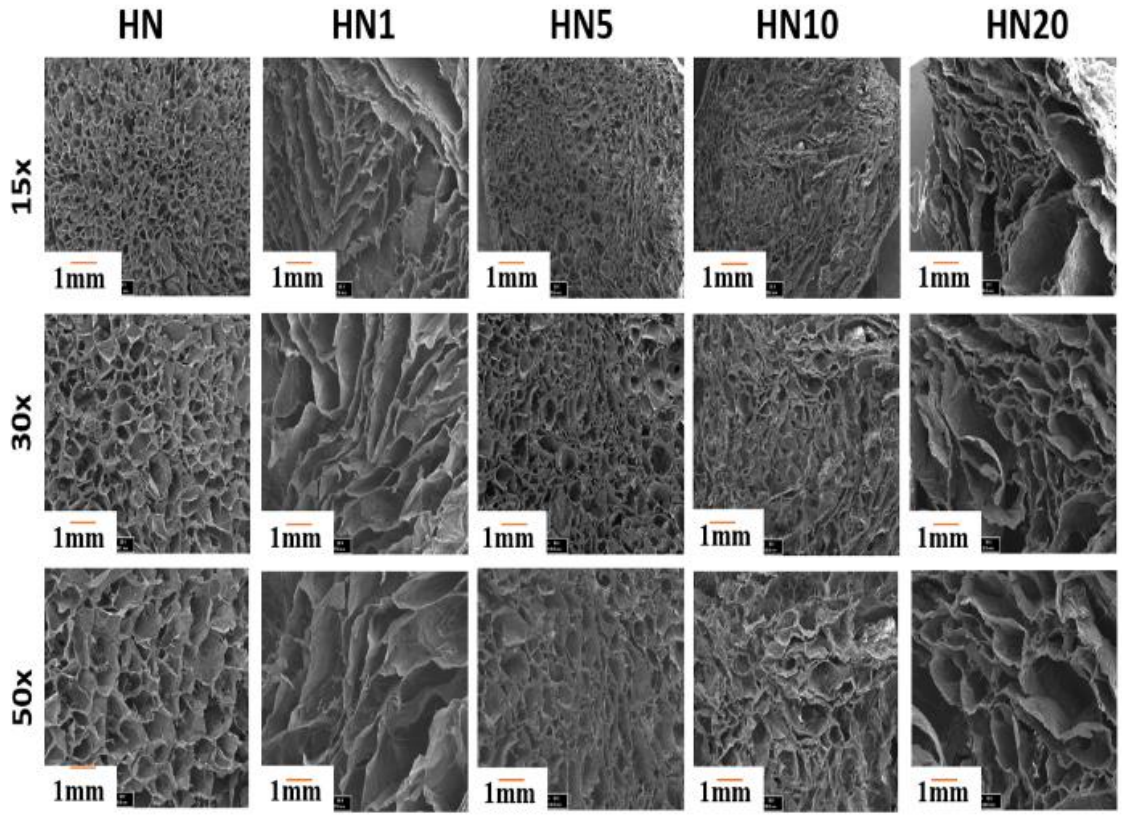


Figure 3.5 SEM images of cryogels containing *V. thapsus*.

3.3.2 Chemical Characterization

The cryogels with and without *V. thapsus* extract were chemically characterized by FTIR, as seen in Figure 3.6. The *V. thapsus* extract showed characteristic peaks at 3323 cm^{-1}, 2923 cm^{-1}, 2853 cm-^{1}, 1624 cm^{-1}, 1405 cm^{-1}, and 1041 cm^{-1}. When compared to the literature's data, the peak at around 3323 cm^{-1} is associated with O-H stretching bonds of alcoholic and phenolic groups of the extract while the peaks at 2923 cm^{-1} and 2853 cm^{-1} represent C-H stretching bonds [167]. The peaks observed at 1624 cm^{-1} and 1405 cm^{-1} can be attributed to C=C stretching and O-H bending (carboxylic or alcoholic groups) bonds and the peak at 1041 cm^{-1} represents ester bonds in the extract [168]. The HN without *V. thapsus* extract demonstrated a peak at 3294 cm^{-1}, which corresponded to the N-H stretching in the amines of chitosan and gelatin. Another peak at 1635 cm^{-1} amide I, represents C=O stretching bonds as well as C=N stretching bonds (Schiff's base) that cross-linking amide and aldehyde groups reveal -C=N imine (Shiff base) as releasing a water molecule, while the peak observed at 1543 cm^{-1} wavelength is amide II bond represents the mixed vibrations of N-H bending and C-N stretching bonds that occur with NH_2 deformation. [169]. In addition, the absence of a peak at a wavelength of 1700 cm^{-1}

was evaluated as the successful removal of unbound glutaraldehyde from HN [170]. The success of impregnating extract to HN cryogel was examined and the results demonstrated that increasing the amount of extract in the cryogel showed an increase in the characteristic peak depths of the extract (3323 cm^{-1}, 2923 cm^{-1}, 2853 cm^{-1}, 1624 cm^{-1}, 1405 cm^{-1}, and 1041 cm^{-1}). It was evaluated that the extract impregnation process was carried out successfully.

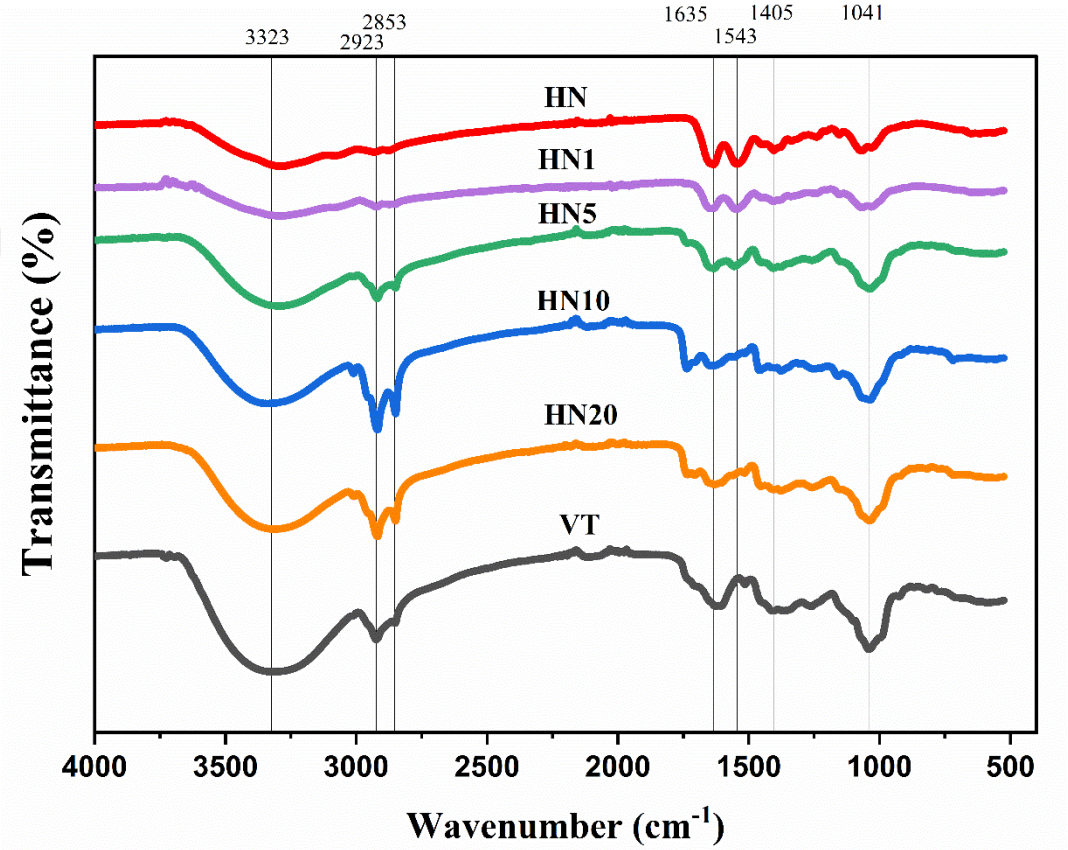


Figure 3.6 FTIR spectrum of *V. thapsus* extract, HN, HN1, HN5, HN10 and HN20 cryogels.

3.3.3 Swelling Capacity

The swelling ability of hemostatic materials is important in controlling bleeding and absorbing exudate from the injury site. Figure 3.7 show the swelling ratio of the cryogels in PBS and citrated blood, respectively. In PBS, the swelling ratio of the cryogels was 3348%, 894%, 674%, 420%, and 312% whereas in blood it was 3647%, 1054%, 438%, 518%, and 313% for HN, HN1, HN5, HN10 and HN20 cryogels, respectively. The addition of *V. thapsus* extract to the cryogels caused a reduction in swelling capacity. This might be because the impregnation of the *V. thapsus* extract altered the pore size and

homogeneity. Similarly, in the literature, adding bio-glass to chitosan cryogel and the dopamine to pullulan hydrogel reduced the swelling ratio due to changes in pore structure [107], [171]. Also, the cryogels swelled rapidly in the first 5 minutes. The rapid swelling and high absorption capacity of hemostatic materials play a crucial role in hemostasis to enhance and accelerate hemostasis [106]. It can be concluded that the cryogels containing *V. thapsus,* having high swelling and blood absorption capacity, are suitable for hemostatic applications.

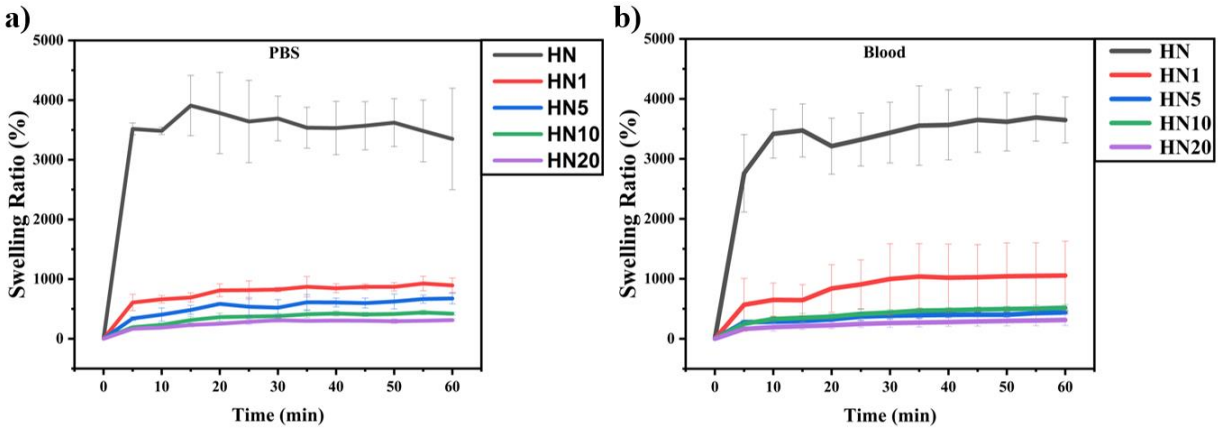


Figure 3.7 Swelling ratio of the cryogels in PBS (n=3), and d) Swelling ratio of the cryogels in blood (n=3).

3.3.4 Mechanical Analysis

Compression test was performed to investigate the mechanical behavior of the cryogels with and without *V. thapsus* extract, and the results were represented in Figure 3.8. The first crack formation started at 4.6±3.07 kPa, 4.5±0.93 kPa, 4.5±0.54 kPa, 5.4±0.88 kPa, and 4.0±0.71 kPa for HN, HN1, HN5, HN10, and HN20, when their strain percentage as 66.8±5.95%, 65.8±2.6%, 64.7±4.92%, 56.8±2.98%, and 56.8±16.65%, respectively. The compression test was continued up to 50 kN force, the cryogels elongated up to 86.00 ± 0.08%, 100.42 ± 7.76%, 89.84 ± 1.80%, 76,25 ± 7.55% and 76.28 ± 6.28% which belong to HN, HN1, HN5, HN10, and HN20, respectively. The cryogels compressive modulus was determined as 0.07±0.04, 0.15±0.07, 0.23±0.05, 1.8±0.56, and 2.65±0.63 kPa for the HN, HN1, HN5, HN10, and HN20 cryogels, respectively. According to these, cryogels were demonstrated similar mechanical behavior, the addition of extract into the cryogel decreased the elongation percentage due to filled voice space in cryogel's pores. On the other hand, extract has the increasing effect on modulus, it may be caused by extract makes a little brittle to the cryogel pore wall. In the literature, when the cryogel's voice space decreased it becomes more concentrated with high

resistance to deformation [148]. According to literature data, a low young modulus between 1-100 kPa is desirable for the biomaterials used in soft tissues [172]. The obtained results showed that the cryogels mechanical properties can meet the requirements for hemostatic applications.

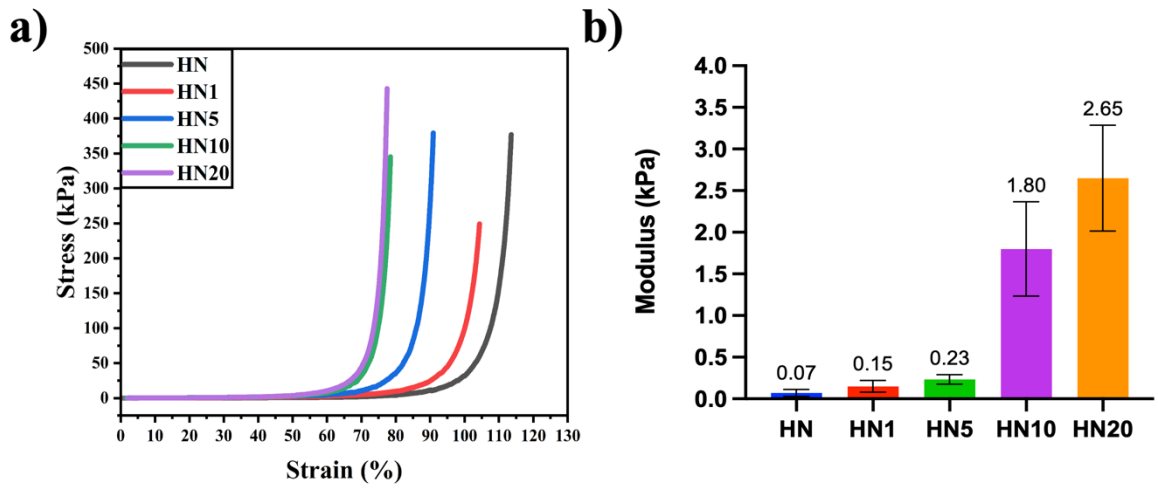


Figure 3.8 a) Stress-Strain curve of the cryogels, b) the compressive modulus (kPa) of the cryogels.

3.3.5 Antibacterial Activity

An ideal hemostatic dressing should prevent bacterial proliferations on the wound site to avoid further wound infections [107]. The bactericidal ratio of fabricated cryogels with *V. thapsus* extract was investigated against *E. coli* and *S. aureus* as shown in Figure 3.9. The bactericidal ratio of the cryogels against *E. coli* was 31.26 ± 7.16%, 71.56 ± 18.39%, 87.85 ± 0.95%, 88.56 ± 0.59%, and 89.51 ± 0.26% whereas against *S. aureus* it was 41.50 ± 2.79%, 70.56 ± 13.65%, 71.38 ± 11.61%, 78.19 ± 0.57%, and 75.22 ± 1.68% for HN, HN1, HN5, HN10 and HN20, respectively. Chitosan is known to have antibacterial activity due to an electrostatic interaction between its polycationic structure and the anionic components of the bacterial surface [173], [174]. Therefore, this adsorption mechanism alters the permeability of the bacterial cell membrane, which leads to membrane disruption and eventually cell death [175]. Although the bactericidal ratio of chitosan/gelatin cryogel (HN) was 31.26 ± 7.16% and 41.50 ± 2.79%, the bactericidal ratio was increased up to ~90% and ~80% with the increase proportionate to the concentration of *V. thapsus* extract against *E. coli* and *S. aureus*, respectively. There are several studies in the literature regarding the antibacterial activity of the biochemical

compounds found in *V. thapsus* extract. It was explained that the antibacterial activity of *V. thapsus* is attributed to phytochemicals such as hexadecenoic acid, undecanol, 9,12-octadecadienoic acid, methyl ester, and (9Z,12Z,15Z)-octadeca-9,12,15-trien-1-ol phytochemicals [176]. Additionally, it is known that fatty acids like 16-carbon chained palmitic acid and 18-carbon chained stearic acid has antibacterial activity against gram-negative and gram-positive bacteria [160]. Moreover, saturated or unsaturated fatty acids including lauric acid, tetradecanoic acid, 3-hydroxyundecanoic acid, 2-hydroxytetradecanoic acid, 9-hexadecenoic acid, 7-hexadecenoic acid, hexadecanoic acid, 10-methylenehexadecanoic acid, heptadecanoic acid, 12-octadecadienoic acid, cis-9-octadecadienoic acid, stearic acid and cis-9, 10-methyleneoctadecanoic acid create protonophore effect due to their acidic groups, thereby increasing the bactericidal effect [161], [177]. The antibacterial activity of (Z) 6, (Z) 9-pentadecadien-1-ol, which is fatty acid alcohol, and hexadecanoic acid methyl ester has been also mentioned in the literature. [178], [179], [180]. Another research showed that hexadecanoic acid increases membrane fluidity by reducing the polarization index in the bacterial membrane which results in a decrease in ATP level and cell death [177]. Also, it has been found that the aldehydes such as hexanal react with functional groups like the sulphydryl group altering the permeability of the bacterial membrane and exerting an antimicrobial action against fungi and bacteria [159]. Not only the *V. thapsus* extract, but also the *V. thapsus* plant oil demonstrated antibacterial effect against 4 different bacterial species and 2 different fungus species in the literature [162]. In conclusion, the antibacterial activity of the *V. thapsus* extract can be explained by the compounds that were found by GC-MS analysis to cause bacterial death by affecting the functionality, permeability and integrity of the bacterial membrane. [159].

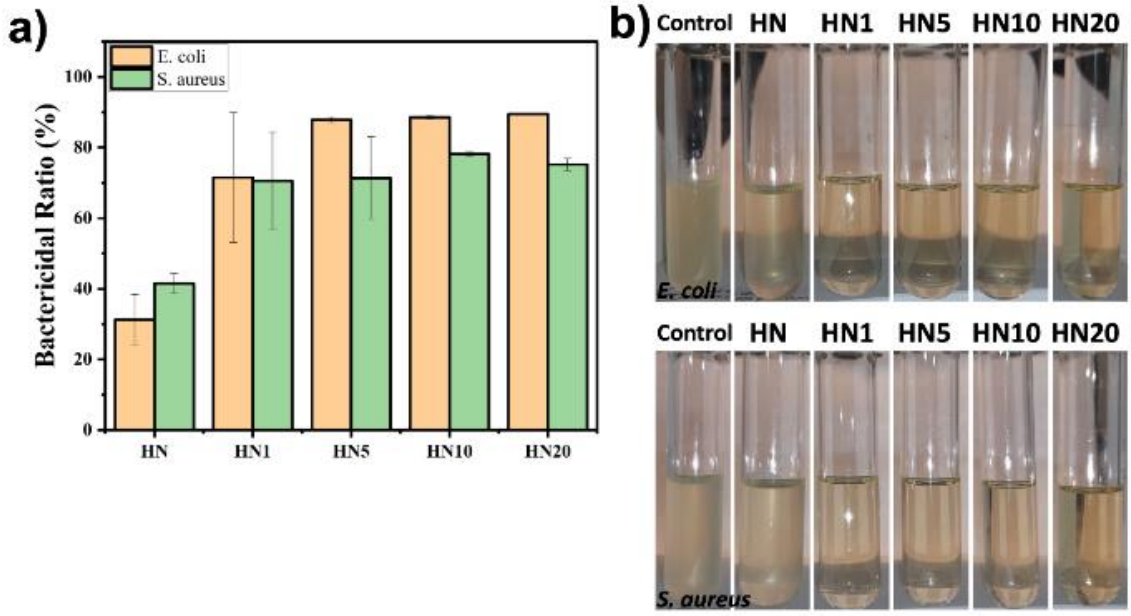


Figure 3.9 a) Bactericidal ratio (%) of HN, HN1, HN5, HN10, and HN20 cryogels against *E. coli* and *S. aureus*, and b) Experimental representation of the cryogels' antibacterial activity (n=3).

3.3.6 *In vitro* Cell Viability

The viability of human dermal fibroblast cells on the cryogels was investigated using MTT assay for 24, 48, and 72 hours, the results were represented graphically in Figure 3.10. At 24 hours, the cryogels had over 94% cell viability, whereas HN5 had 105% cell viability compared to the control group. At 48 hours, HN, HN1, HN5, and HN10 cryogels had cell viability of more than 100%, whereas HN20 had 84% cell viability. Furthermore, HN, HN1, HN5, HN10, and HN20 cryogels demonstrated 75%, 95%, 94%, 69%, and 58% cell viability for 72 hours, respectively. Chitosan and gelatin, both biocompatible polymers, were used in this study, and low concentration of glutaraldehyde had no toxic effect on fibroblast cell line [121]. In the literature, a study confirmed that 1% and 0.3% (w/v) glutaraldehyde for chitosan/gelatin cryogels had no cytotoxicity [119]. Furthermore, the methanolic extracts of several *Verbascum* species were investigated for their effects on cells, and no cytotoxic effect on fibroblast cells was seen. Also, they reported that the low concentrations of *V. thapsus* extract had a proliferative effect on fibroblast cells, while the high concentrations have an adverse effect on cell viability [181]. *Verbascum* plants and their methanolic extracts have antioxidant effects because they contain iridoid/neolignane type glycosides, oleanan type terpenes, flavonoids, polysaccharides, saponins, steroids, and alkaloids which have

several biological properties such as antimicrobial, anti-inflammatory and antioxidant activities by stimulating endogenous antioxidant mechanisms that protect from and scavenge free radicals, thereby decreasing oxidative damage [182], [183]. Also, antioxidant activity of *V. thapsus* phenolic compounds has been reported in the literature [184]. Because of the considerable antioxidant compounds found in *V. thapsus* extract, different forms of delivery systems are prepared for the treatment of lung diseases or other degenerative disorders due to antioxidant phytochemicals [185]. Therefore, *V. thapsus* extract could be used at specific concentrations to scientifically benefit even though it is a commonly used folk medicine for a variety of uses. Lower concentrations of *V. thapsus* extract as seen in the HN1 and HN5 cryogels with higher cell viability can be used safely. Furthermore, the cryogels are designed to be used for transient pre hospital care, so none of the cryogels would perform toxic effect during usage.

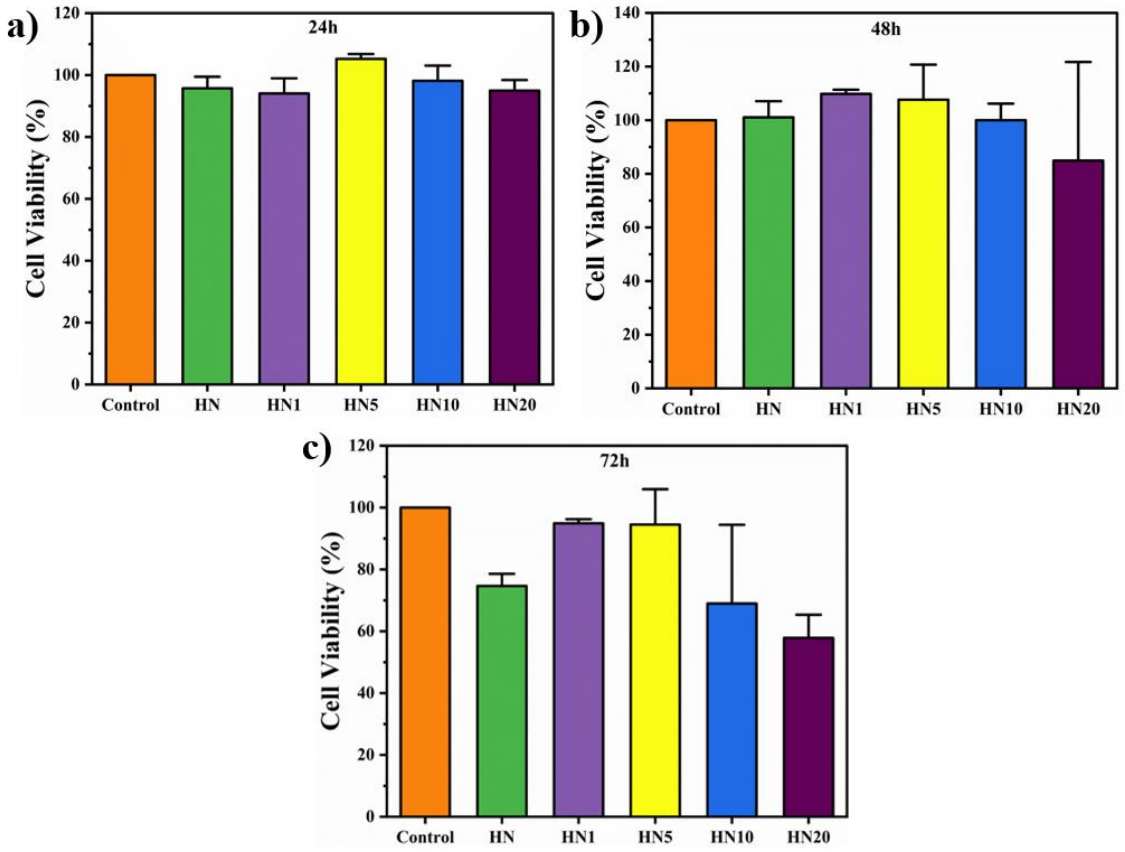


Figure 3.10 The cell viability (%) of the HN, HN1, HN5, HN10 and HN20 cryogels on the human dermal fibroblast cell line for a) 24h, b) 48h, c) 72h.

3.4 Hemostatic Activity of the Cryogels Containing *V. thapsus*

According to the literature, the major plant families Compositae, Lamiaceae, Fabaceae, and Asteraceae, which contain tannins, iridoid glycosides, glycoconjugates, lignans, saponins, and phenolic compounds trigger coagulation by increasing factor XII activity, plasma fibrinogen levels, vascular or smooth muscle contraction, and platelet aggregation while inhibiting fibrinolysis [186]. In this study, the hemostatic effect of the *V. thapsus* plant extract was examined when combined with chitosan/gelatin cryogels, and their hemostatic properties were reported.

3.4.1 Hemolysis Assay

Hemolysis, also known as the rupture of red blood cells (RBCs) is triggered by the release of hemoglobin thus a rise in free hemoglobin concentration in plasma is a sign for the destruction of RBCs. Damage to erythrocytes can reduce oxygen delivery to tissues and organs leading to toxicity. [187]. The hemolysis assay, which quantifies the free hemoglobin calorimetrically, was used to investigate the effect of cryogels containing *V. thapsus* extract on RBCs and their blood compatibility. The hemolysis test was applied based on the ISO 10993:4 standard. Since normal saline is isotonic to blood serum, the negative control had no effect on the RBCs, whereas distilled water, the positive control, disrupted the RBCs by hemolysis and thereby freed the hemoglobin. Figure 3.11 shows the hemolysis ratio of the HN, HN1, HN5, HN10 and HN20 cryogels as 1.02 ± 0.003%, 1.01 ± 0.004%, 1.01 ± 0.004%, 1.00 ± 0.006%, 1.01 ± 0.006%, respectively. These results demonstrated that the whole cryogels are blood compatible because of having approximately ~1% hemolysis ratio. According to ASTM F756-08 standard, medical devices in contact with blood with a hemolysis ratio lower than 5% can be accepted as safe for medical use. [188] In the literature report, *V. thapsus* extract hemocompatibility was reported, aqueous and methanolic extracts of *V. thapsus* roots demonstrated the 4.39 % and 3.11% hemolysis, respectively [184]. In another study, hemolysis ratio of *V. thapsus* aqueous extract was reported as 1.2% [189]. It was reported that the chitosan or gelatin-based scaffolds also showed blood compatibility in the literature. [190], [191]. It

was assessed that the fabricated cryogels with *V. thapsus* extract are blood compatible and can be accepted safe for medical use, also obtained results are consisted with literature's data.

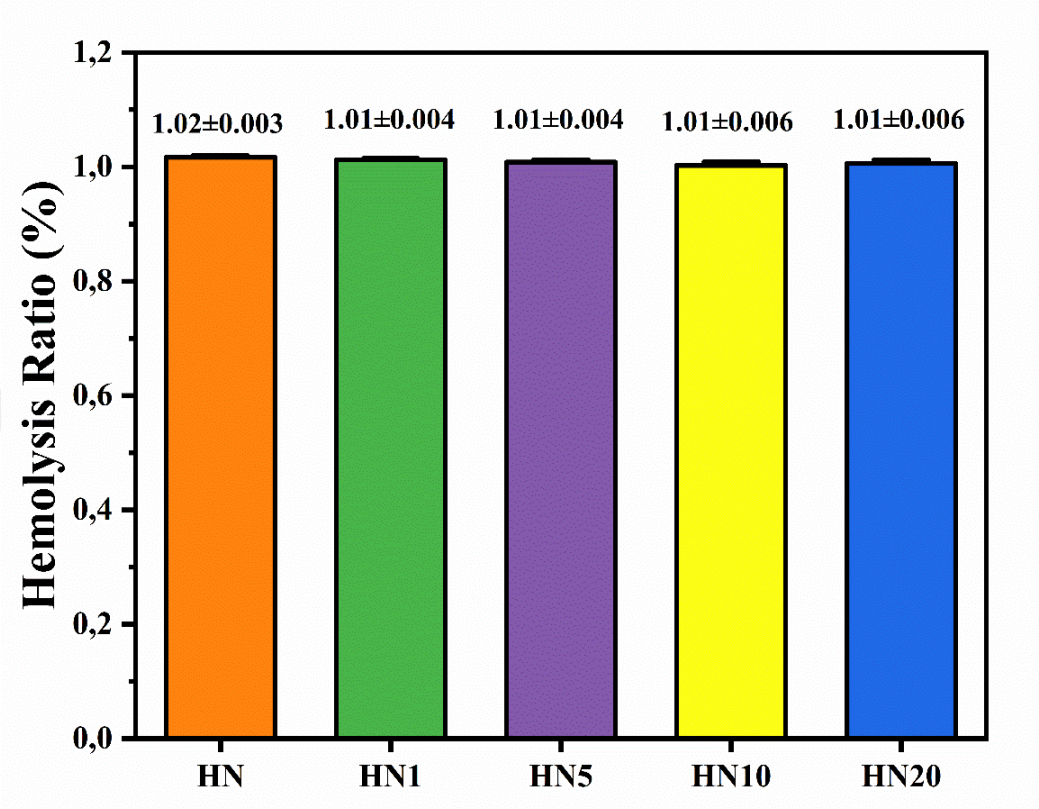


Figure 3.11 The hemolysis ratio of cryogel samples (n=3).

3.4.2 *In vitro* Whole Blood Clotting

The aim of blood clotting research is to determine whether the hemostatic material promotes blood clot formation, measured using BCI to assess its performance. A lower BCI value indicates a greater ability of the material to promote clotting [133]. The blood coagulation capacity of the cryogels was evaluated by *in vitro* whole blood clotting assay as represented in Figure 3.12, and the BCI was calculated. The distilled water was used as a reference which disrupted the red blood cells and released hemoglobin, causing the solution to appear red, as seen in Figure 9a. The cryogels and commercial blood stopper demonstrated good blood coagulation properties within 5 minutes as clearly seen in Figure 3.12.a. The cryogels demonstrated the close coagulation ability to the commercial blood stopper material. This can be explained by the positive charges of chitosan interacting with the negatively charged blood cells [192]. Figure 3.12.b represents the

BCI values of commercial blood stopper, HN, HN1, HN5, HN10 and HN20 which were 5.94 ± 0.57, 11.92 ± 1.49, 11.31 ± 2.44, 6.82 ± 0.31, 6.46 ± 1.03 and 5.86 ± 0.61, respectively.

According to blood clotting tests, the increasing *V. thapsus* concentration from 1% to 20% in cryogels improved the blood clotting activity significantly. This can be explained by the fatty acids in *V. thapsus* extract triggering blood coagulation pathways [179]. In the literature, the BCI value of traditional dressing materials was found to be 66.3 ± 5.5% for cotton yarns. [166]. Blood clotting can be related to the contact area, fabric structure, porosity and blood absorption capacity. Thus, a higher porous structure could be beneficial for more absorption of blood, resulting in a lower BCI value [166]. It can be concluded that the *V. thapsus* containing cryogels have good potential as a hemostatic material compared to the traditional materials such as cotton yarns.

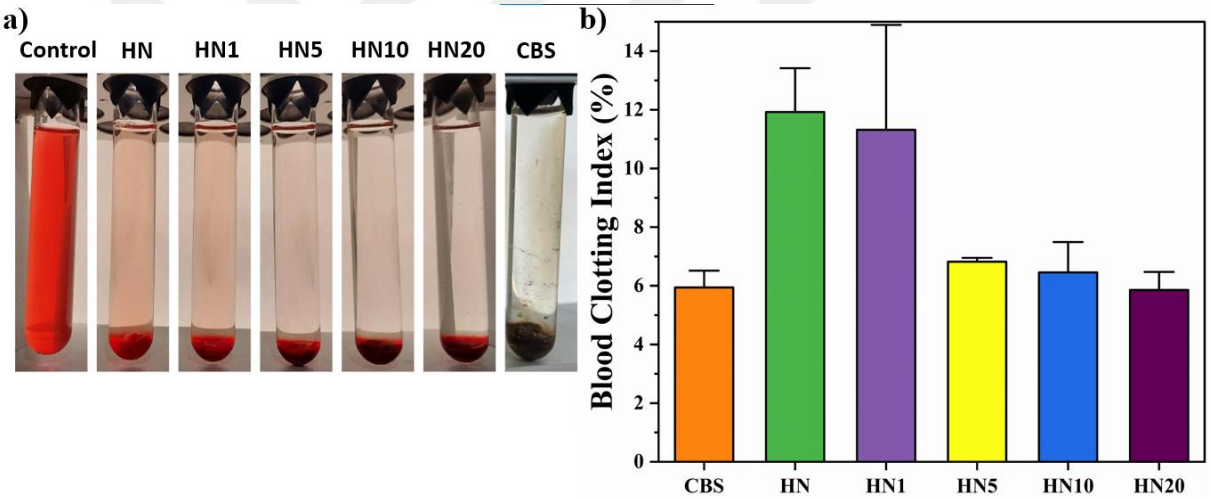


Figure 3.12 a) Experimental representation of the whole blood clotting assay, b) graphical representation of the whole blood clotting index (n=3).

3.4.3 *In vitro* Whole Blood Clotting Time

The blood clotting time is another important criterion for hemostatic materials to achieve immediate blood stoppage after trauma or injury. The blood clotting times of samples and commercial blood stopper was investigated at intervals of 1, 3, 5, 10, 15, 20, 25 and 30 minutes and presented in Figure 3.13. The HN10, HN20 and CBS demonstrated the better blood clotting properties then HN and HN1, attributed to the increased concentration of *V. thapsus* in the cryogel which improved the blood clotting time of the cryogels. The blood clotting times of the samples were graphically represented in Figure 3.13.b. In the control group (empty well), blood clotting started at 9.95 ± 0.4 minutes. HN

and HN1 showed similar clotting times to the control, which were 9.57 ± 1.42 and 8.70 ± 0.47 minutes, respectively. Indeed, HN5, HN10, and HN20 (6.4 ± 0.06, 6.15 ± 0.51 and 6.14 ± 0.51, respectively) demonstrated faster blood clotting than CBS which was 7.42 ± 0.17. This can be explained by the phytochemicals of *V. thapsus* extract, which may improve blood coagulation. *V. thapsus* contains several fatty acids, especially stearic acid, which can play important role in blood coagulation by activating the Factor XII in the coagulation pathway and promoting platelet aggregation [179]. The phenolic and flavonoid components of *Verbascum* species also play important roles in the coagulation cascade. Phenols accelerate the interaction between thrombin and fibrinogen [193]. The synergistic interaction between the molecules and plants can be correspond to the bioactivity of plants [193]. On the other hand, material design is an important parameter for achieving effective hemostatic properties. Similar clotting times were seen in Zhang et al.'s study, where they increased the thickness of 3D knitted materials, and reduced blood clotting time from 10.12 ± 0.11 minutes to 8.16 ± 0.17 minutes. [166]. The obtained results were consistent with the data reported in the literature [190], suggesting that *V. thapsus* extract in chitosan/gelatin cryogels are promising candidates for hemostatic applications.

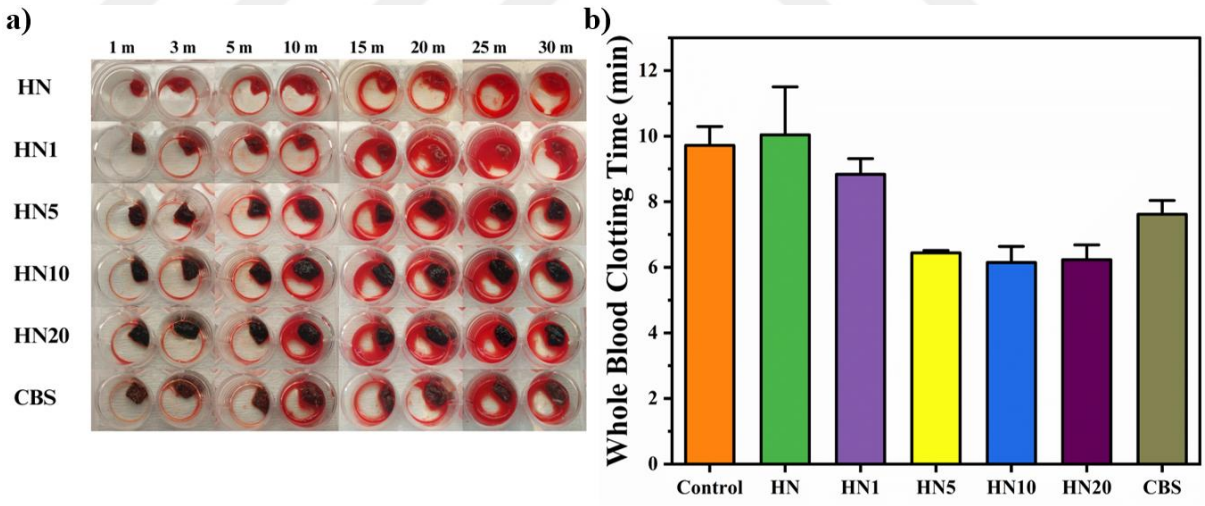


Figure 3.13 Experimental representation of the whole blood clotting time, and d) graphical representation of the whole blood clotting time for the HN, HN1, HN5, HN10, HN20 and CBS (commercial blood stopper) (n=3).

3.4.4 Platelet Adhesion

In the blood coagulation pathway, the platelet aggregation and blood cell activation through the adherence on the surface are important factors for hemostasis [194]. The platelet adhesion assay assessed the interaction between red blood cells and cryogel samples, as well as the cryogels' ability to promote aggregation. The platelet adhesion on the cryogels by DAPI staining was represented in Figure 3.14.a. Firstly, the whole blood was added on the cryogels and was absorbed quickly because of high swelling capacity which help primary hemostasis by concentrating the coagulation factors accelerating the coagulation cascade and afterward a blood clot is formed [195]. Then, the cryogel provides surface and macropores for blood cell adhesion. Positively charged surface of the cryogel aggregates negatively charged platelets and *V. thapsus* extract activate Factor XII to trigger the intrinsic pathway which eventually leads to the production of fibrins [186], [196]. As shown in Figure 3.14.a, platelet adhesion on the cryogels is increased with the addition of *V. thapsus* extract, it can be explained by the tannin and flavonoids content of *V. thapsus* increased blood cell adhesion[197]. It may be due to these compounds can improve the surface characteristics of a material, making it advantageous to platelet adhesion. As the concentration of plant extract within the cryogel increases, the availability of bioactive chemicals is also increased, resulting in more interactions with platelet, thereby increased platelet aggregation, adhesion, and activation, promoting enhanced hemostasis [19]. The blood cells adhered thoroughly and linked with one another. A fibrin clot was found among the blood cells and cryogel. According to the results investigated from the experiments, HN5 sample was chosen in terms of good swelling capacity, low cell cytotoxicity, and favorable hemostatic efficiency which is an excellent candidate for being hemostatic dressing material. Therefore, the platelet adhesion on HN5 was examined by SEM and represented in Figure 3.14.b. The aggregation and adherence of red blood cells, and others can be clearly seen on the surface of HN5. The HN5 was shown to be efficient in aggregating red blood cells and promoting fibrin formation [129].Therefore, the cryogel absorbs blood from the injury site and physically seals the wound while initiating and accelerating the coagulation cascade through physiological, biological and physical mechanisms. According to the results investigated from the experiments, HN5 sample was chosen in terms of good swelling capacity, low cell cytotoxicity, and favorable hemostatic efficiency which is an excellent candidate for being hemostatic dressing material

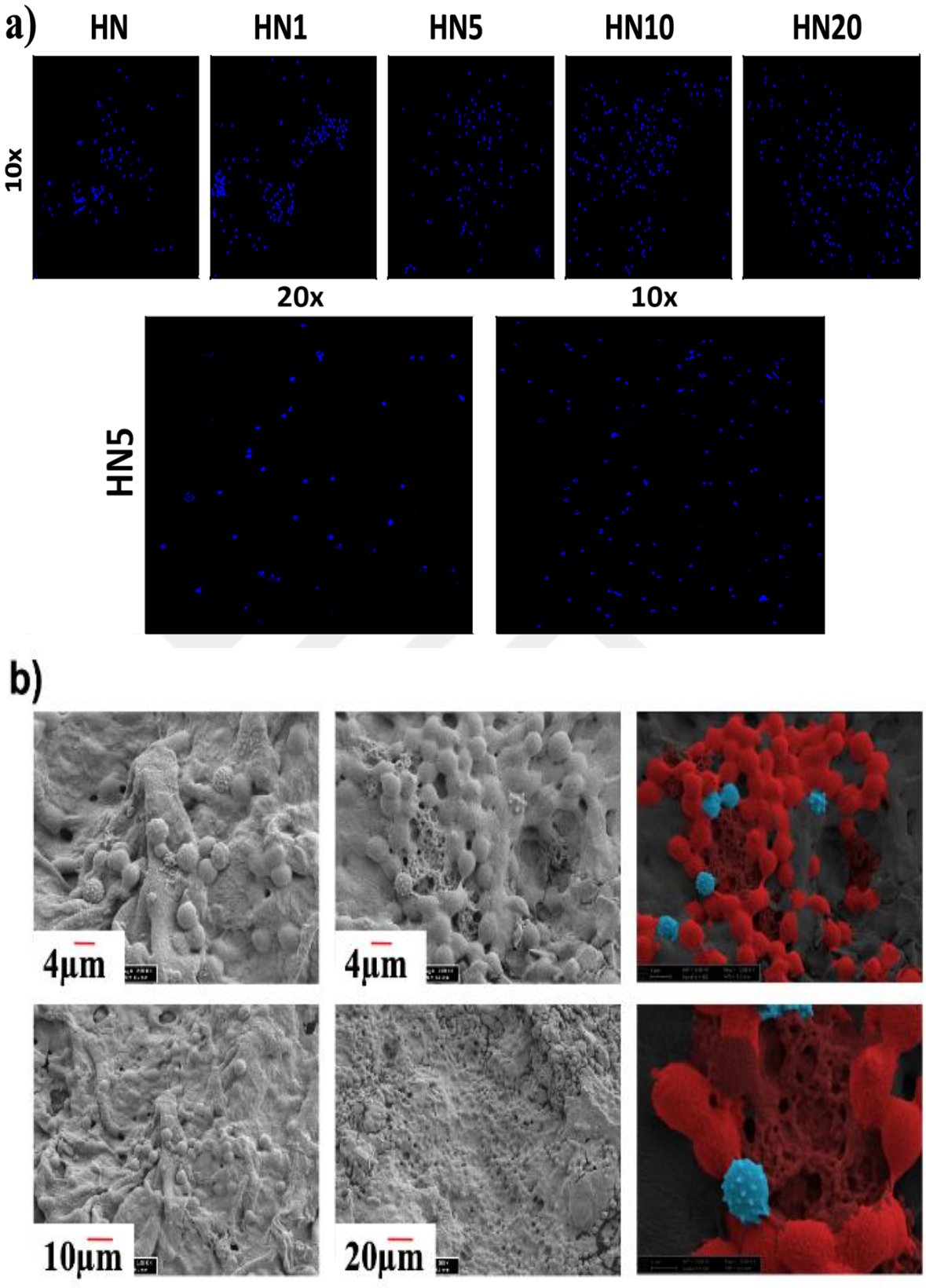


Figure 3.14 a) DAPI staining of HN, HN1, HN5, HN10, and HN20 cryogels and b) the SEM images of platelet adhesion and clotting formation on HN5 cryogel.

Chapter 4

Conclusions and Future Prospects

4.1 Conclusions

In this study, we developed a novel hemostatic biomaterial by chitosan/gelatin cryogel containing *Verbascum thapsus* extract. Chemical crosslinking of chitosan and gelatin by glutaraldehyde at -20 °C and freeze-drying methodology was used to achieve macroporous scaffold structure. Based on morphological and physical characterizations, the suitable chitosan/gelatin cryogel which has a more homogeneously distributed pore structure with thinner pore walls between 200-250 μmand higher swelling ratio of about 3350% was optimized among the samples having a variety of chitosan: gelatin ratios, and crosslinker concentrations. Further with the impregnation of *V. thapsus* extract at different concentrations, the cryogels were characterized morphologically by SEM, physically by swelling behavior, chemically by FT-IR analysis, and mechanically by compression analysis. The results demonstrated that the cryogels have a suitable macroporous structure with high swelling ability in PBS and citrated blood, the crosslinking and impregnation of the extract were chemically successful, and the cryogels have mechanical strength appropriate for soft tissue applications. Furthermore, the cryogels exhibited bacterial inhibitions proportional to extract concentration, up to 89% and 78% bactericidal ratio was observed against *E. coli*, and *S. aureus*, respectively. The cell viability of the cryogels was investigated by a human fibroblast cell line which showed more than 90% cell viability for 24 hours and 48 hours, even though a higher concentration of the extract decreased cell viability after 48 hours. Finally, the hemostatic efficiency of the cryogels was investigated. Whole blood clotting assay showed that the BCI of the chitosan/gelatin cryogels was improved from 11.9 to 6.5 and the clotting time was decreased with the addition of *V. thapsus* extract. Also, platelet adhesion on the cryogels is increased as the

concentration of plant extract within the cryogel increases. Enhanced hemostatic activity of the chitosan/gelatin cryogels can be explained by the bioactive phytochemicals in the extract increasing interactions with platelets, thereby increasing platelet adhesion and aggregation, and triggering coagulation cascade.

In conclusion, the chitosan/gelatin cryogel impregnated with VT plant extract is a promising hemostatic biomaterial owing to its unique properties. Cryogels' interconnected macroporous structure allows for high fluid absorption, promotes rapid blood clotting, and facilitates cell infiltration, making them extremely effective in controlling bleeding. Chitosan and gelatin contribute to the material's bioactivity through their innate properties and functional groups which have critical roles in hemostasis by forming electrostatic interactions with blood components, and improving platelet adhesion, activation, and aggregation accelerating the coagulation process. The addition of *Verbascum thapsus* extract improves the hemostatic performance of the cryogel. The phytochemicals of the extract, which are known for their bioactive qualities in hemostasis, significantly enhance the material's ability to stimulate the coagulation cascade, resulting in accelerated clot formation while also enhancing the antibacterial effect of the plain scaffold against both gram positive and negative bacteria which is essential to prevent infections at wound site. The plant extract and cryogel matrix collaborate to increase bioactivity while also improving the material's overall efficiency in encouraging rapid bleeding control. This unique combination has enormous promise for use in advanced hemostatic applications, providing a more efficient and bioactive alternative for bleeding control.

4.2 Societal Impact and Contribution to Global Sustainability

The development of a hemostatic chitosan/gelatin cryogel containing *Verbascum thapsus* extract meets crucial demands of society in healthcare, particularly in trauma care and wound management. Severe bleeding is one of the leading causes of death

worldwide, particularly in trauma and surgical situations. This initiative helps to develop more sustainable, biocompatible, cost-effective and efficient medicinal solutions addressing the demand for advanced wound care, and improving patient outcomes. By incorporating natural resources and biodegradable materials into healthcare, this project not only advances the field of medicine but also demonstrates sustainable innovation. Therefore, it outlines a strategy for developing environmentally responsible biomaterials that address global health and sustainability issues.

This strategy supports the United Nations Sustainable Development Goals (SDGs). It aligns particularly with SDG 3, which is Good Health and Well-Being by improving healthcare outcomes, advancing medical innovation, and providing equitable access to essential hemostatic materials. It offers enhanced, easily accessible medical product where access to advanced medical supplies is limited. This study has potential to reduce preventable mortality rates caused by uncontrolled bleeding by reducing bleeding time and improving wound management.

Additionally, the project helps SDG 12, which is Responsible Consumption and Production, by utilizing renewable, biodegradable chitosan and gelatin polymers, reducing reliance on synthetic, and non-biodegradable material alternatives. The use of *Verbascum thapsus* extract also improves sustainability by utilizing natural resources for bioactivity rather than synthetic chemicals, hence lowering its environmental impact.

This project also contributes to SDG 15, which is Life on Land, by addressing the need to preserve biodiversity. Overall, the study demonstrates the production of a sustainable biomaterial that prioritizes environmental awareness and integrates healthcare developments with global sustainability goals.

In conclusion, chitosan/gelatin cryogels containing *Verbascum thapsus* extract meets crucial healthcare needs by preventing bleeding and saving lives, particularly in emergencies and areas with limited resources. Its low-cost and environmentally friendly design promotes accessible, long-term healthcare while supporting innovation and the use of local biodiversity, all of which contribute to better health outcomes and global equity in healthcare.

4.3 Future Prospects

The *Verbascum thapsus*-impregnated chitosan/gelatin cryogel has a promising future as a hemostatic material since it demonstrates the potential of combining natural plant extracts with biomaterials to improve bioactivity and performance. This study contributes to the developing field of biomaterials enhanced with natural sources, providing an environmentally friendly way to improve hemostatic performance. However, more research is required to enhance the composition of the material, addressing potential issues such as variability in plant extract potency and adverse side effects such as allergic reactions or toxicity. Plant extracts' biological complexity requires careful control over purity and dose. Furthermore, while *in vitro* research provides initial findings, *in vivo* investigations remain essential for understanding the material's function and safety within the human body. Long-term biocompatibility, degradation, and immunological response need to be thoroughly investigated before clinical application.

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