Nuray Koç *CENTELLA ASIATICA* EXTRACT CONTAINING BILAYERED ELECTROSPUN WOUND DRESSING AGU 2019

CENTELLA ASIATICA EXTRACT CONTAINING BILAYERED ELECTROSPUN WOUND DRESSING

A THESIS
SUBMITTED TO BIOENGINEERING
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

By
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I hereby declare that all information in this document has been obtained in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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M.Sc.thesis entitled *CENTELLA ASIATICA* EXTRACT CONTAINING BILAYERED ELECTROSPUN WOUND DRESSING has been prepared in accordance with the Thesis Writing Guidelines of the Abdullah Gül University, Graduate School of Engineering & Science.

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Prepared By

Nuray Koç

Advisor

Assist. Prof. İsmail Alper İşoğlu

Head of Bioengineering Program

Prof. Sevil Dinçer İşoğlu

Signature

ACCEPTANCE AND APPROVAL

M.Sc.thesis entitled *CENTELLA ASIATICA* EXTRACT CONTAINING BILAYERED ELECTROSPUN WOUND DRESSING prepared by Nuray Koç has been accepted by the jury in the Bioengineering Graduate Program at Abdullah Gül University, Graduate School of Engineering & Science.

…….. /…….. / ……..

(Thesis Defense Exam Date)

JURY:

Advisor : Assist. Prof. İsmail Alper İşoğlu

Member : Assist. Prof. Ömer Aydın

Member : Assist. Prof. Aysun Adan

APPROVAL:

The acceptance of this M.Sc.thesis has been approved by the decision of the Abdullah Gül University, Graduate School of Engineering & Science, Executive Board dated ….. /….. / …….. and numbered ……..……. .

…….. /…….. / ……..

(Date)

Graduate School Dean

Prof. Dr. İrfan Alan

ABSTRACT

CENTELLA ASIATICA EXTRACT CONTAINING BILAYERED ELECTROSPUN WOUND DRESSING

Nuray Koç
MSc. in Bioengineering
Supervisor: Assist. Prof. İsmail Alper İşoğlu

December 2019

Innovative and bioactive wound dressings prepared by electrospinning mimicking the native structure of the extracellular matrix (ECM) have gained significant interest as an alternative to conventional wound care applications. In this study, bilayered wound dressing material was produced by sequential electrospinning of quaternized poly(4-vinyl pyridine) (upper layer) on the *Centella Asiatica* (CA) extract containing electrospun poly(D, L-lactide-co-glycolide) (PLGA)/poly(3-hydroxybutyrate-co-3-hydroxy valerate) (PHBV) blend membrane (lower layer). Scanning electron microscopy (SEM) was utilized to show a uniform and bead-free fiber structure of electrospun membranes. The average diameter of CA extract containing electrospun PLGA/PHBV blend membrane was calculated 0.471±0.11 μm, whereas the average fiber diameter of electrospun poly(Q-VP) membranes was in the range of 0.460±0.057 μm. Chemical, thermal, mechanical properties, and adsorption capacity of electrospun membranes, as well as the cumulative release of CA from the electrospun PLGA/PHBV membrane, were investigated. Viability, adhesion, and attachment of human fibroblast cells on the electrospun membranes on pre-set days were evaluated by the colorimetric CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS assay) and SEM. Results revealed that CA loaded bilayered electrospun wound dressing showed promoted attachment and proliferation of fibroblasts. Hence, it can be concluded that CA extract containing bilayered electrospun wound dressing prepared in this study has a promising potential for wound healing applications.

Keywords: Wound dressing, Electrospinning, Bilayered, Centella Asiatica

ÖZET

CENTELLA ASIATICA EKSTRAKTI İÇEREN ÇİFT KATMANLI ELEKTROEĞRİLMİŞ YARA ÖRTÜSÜ

Nuray Koç
Biyomühendislik Yüksek Lisans
Tez Yöneticisi: Dr. Öğr. Üyesi İsmail Alper İşoğlu

Aralık 2019

Elektroeğirme yöntemi ile hazırlanan, ekstra sellülar matrisin doğal yapısını taklit eden inovatif ve biyoaktif yara örtüleri geleneksel yara bakım uygulamalarına alternatif olarak önemli bir ilgi kazanmaktadır. Bu çalışmada, elektroeğirme yöntemi ile kuaterner poli(4-vinil piridin) bir üst katmana, *Centella Asiatica* (CA) ekstratı içeren poli(D,L-laktik-ko-glikolik asit) (PLGA)/poli(3-hidroksibütirat-ko-3-hidroksi valerat) (PHBV) bir alt katmana sahip çift katmanlı bir yara örtüsü üretilmiştir. Elektroeğrilmiş membranların uniform ve boncuksuz fiber yapıları taramalı elektron mikroskop (SEM) kullanılarak gösterilmiştir. CA ekstraktı içeren PLGA/PHBV membranların ortalama fiber çapları 0,471±0,11 μm olarak hesaplanırken, elektroeğrilmiş poli(Q-VP) membranların ortalama fiber çapları 0,460±0,057 μm olarak bulunmuştur. Kimyasal, termal ve mekanik özellikleri, elektroeğrilmiş membranların absorbsiyon kapasitesi ve ayrıca PLGA/PHBV membranlardan CA ekstraktının kümülatif salımı araştırılmıştır. Elektroeğrilmiş membranlar üzerinde insan fibroblast hücrelerinin önceden belirlenen günler için canlılık, yapışma ve tutunma testleri colorimetric CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) ve SEM ile gösterilmiştir. Sonuçlar, CA içeren çift katmanlı elektroeğrilmiş yara örtüsünün insan fibroblast hücrelerinin tutunma ve çoğalmasına olanak sağladığını göstermiştir. Bu sebepten, CA ekstraktı içeren çift katmanlı yara örtüsünün yara bakımı uygulamaları için umut verici bir potansiyele sahip olduğu değerlendirilmiştir.

Anahtar Kelimeler: Yara Örtüsü, Elektroeğirme, Çift Katman, Centella Asiatica

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To My Family

Chapter 1

1 Introduction

1.1 Skin Tissue

Skin is the largest organ in the body, which consists of tissues that work together as a single structure, to act as an anatomical barrier against to the toxic substances, pathogens, and organisms and to provide homeostasis, self-healing, and sensory. The skin is approximately 15% of body weight and possesses two main layers: the epidermis, which is composed of epithelial cells and protects the body against external factors, and the dermis, which is made of dense, irregular connective tissue hosting blood vessels, hair follicles, sweat glands, and other structures [1-3]. Dermis ensures the skin elasticity and strength, and also regulates body temperature through control of blood flow and sweating. Epidermis houses keratinocytes as the major cellular element, while dermis possesses the fibroblasts as the main cell type. Under the dermis lies the subcutaneous layer (the hypodermis), which is composed mainly of loose connective and fatty tissues. The deeper layer of skin is well vascularized (has numerous blood vessels). It also has numerous sensory, and autonomic and sympathetic nerve fibers ensuring communication to and from the brain (Figure 1.1.1) [4].

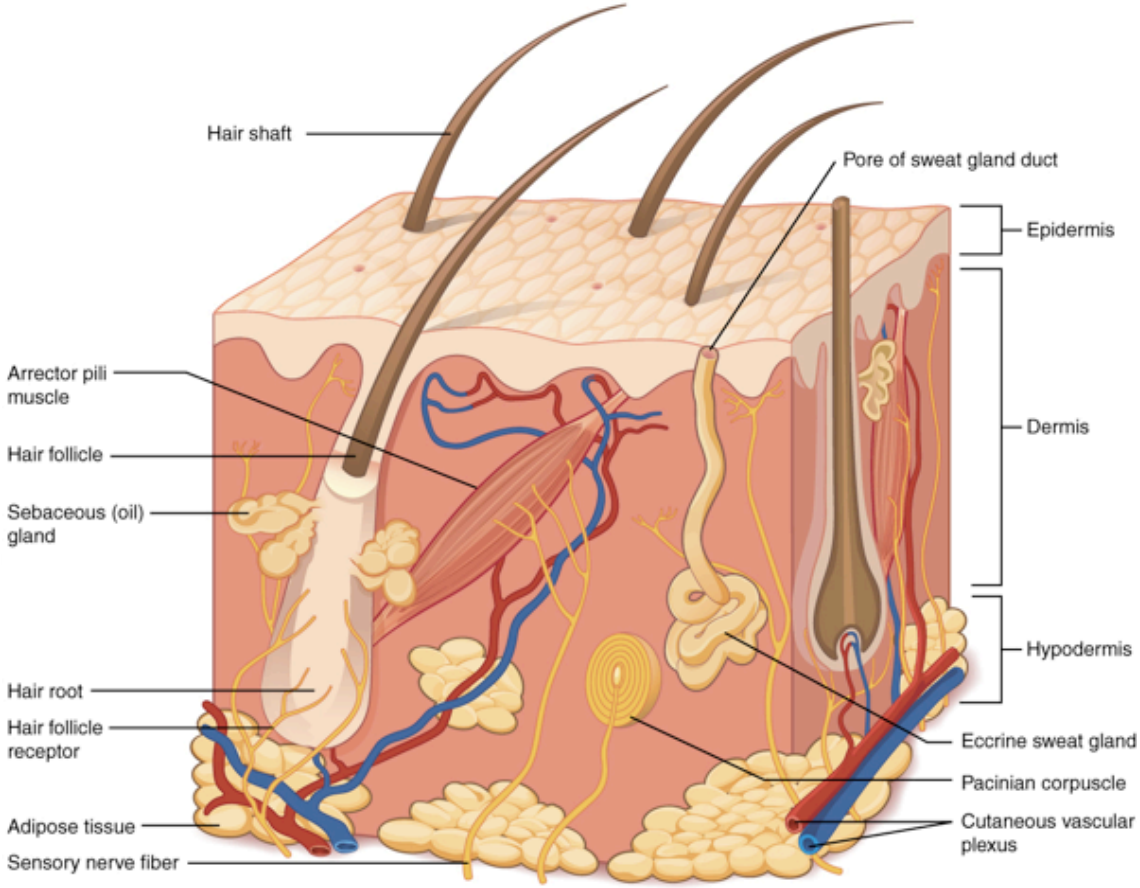

Figure 1.1.1 Layers of Skin [4].

1.1.1 The Epidermis

The epidermis is consisted of five layers of epithelial cells, which are stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum, without any blood vessels (Figure 1.1.1.1). The major cell type in epidermis is a keratinocyte that manufactures and stores the protein keratin, an intracellular fibrous protein giving hair, nails, and skin their hardness and water-resistant properties [4].

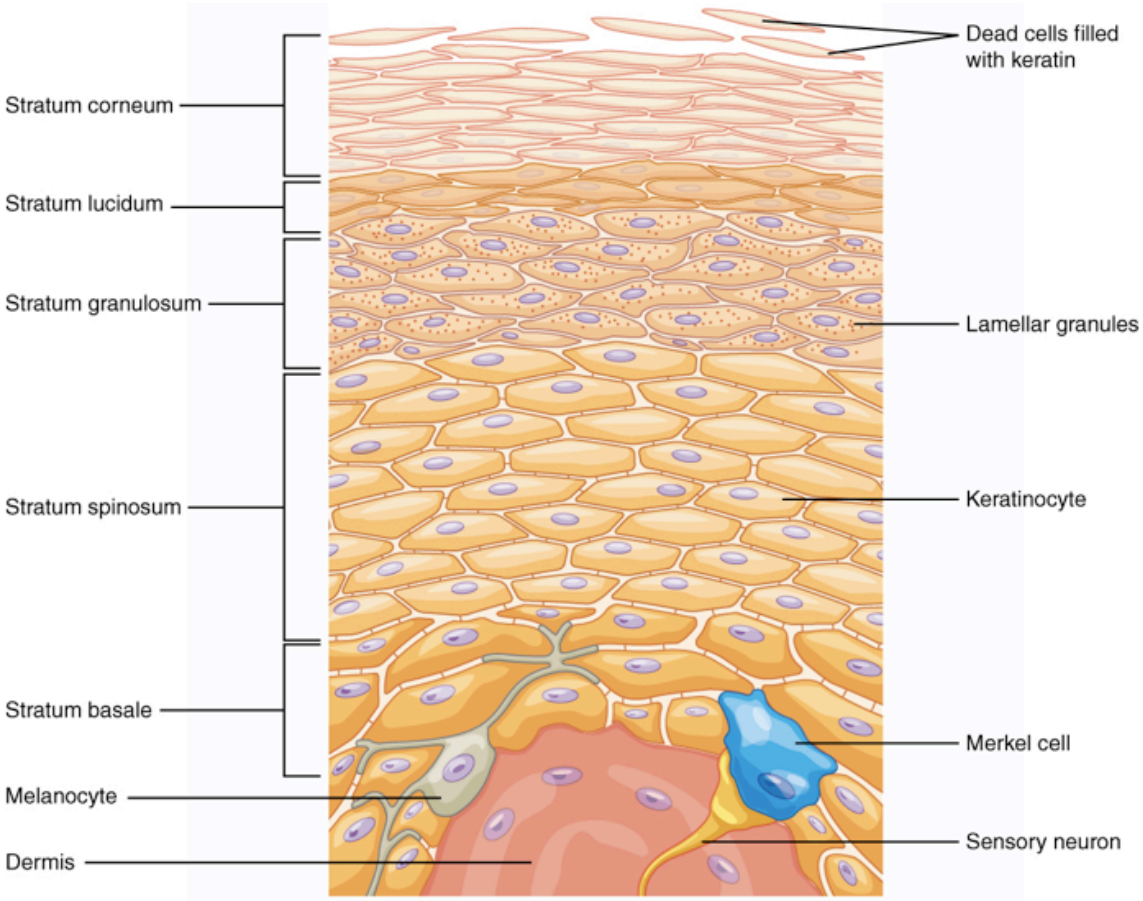

Figure 1.1.1.1 Layers of Epidermis [4].

1.1.2 The Dermis

The dermis has two components of connective tissue, which are the papillary layer and reticular layer. Both are made of an interconnected mesh of elastin and collagenous fibers produced by fibroblasts. It also stores blood and lymph vessels, nerves, and other structures, such as hair follicles and sweat glands (Figure 1.1.2.1) [4].

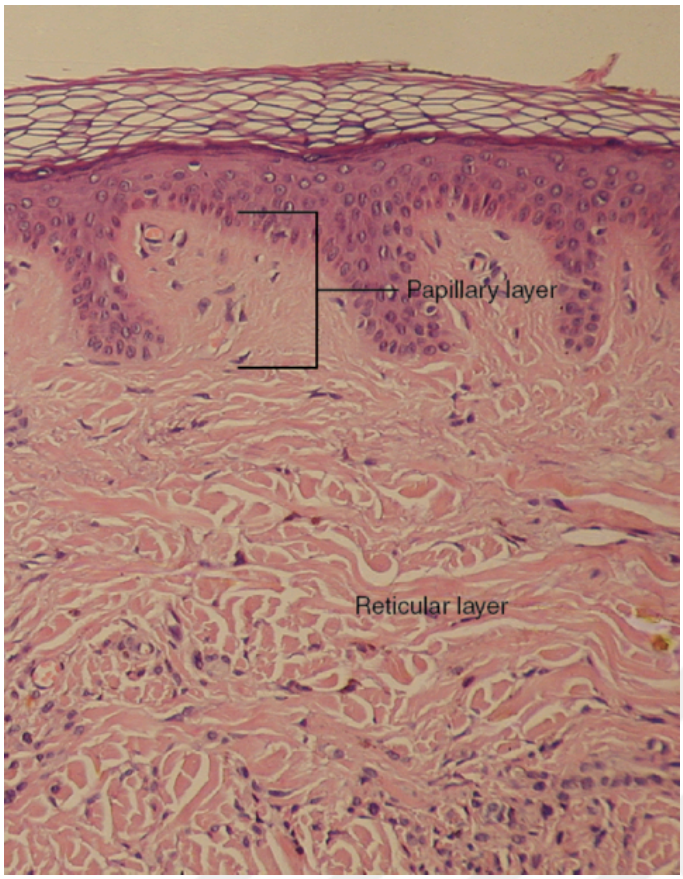

Figure 1.1.2.1 Layers of Dermis [4].

1.1.3 The Subcutaneous Layer

The subcutaneous layer (also called the hypodermis) is a layer beneath the dermis and attaches the skin to the underlying the bones and muscles. The subcutaneous layer is made of well-vascularized, loose, areolar connective tissue, and adipose tissue consisting of adipocytes, the cells that store the fat that insulates the body to prevent heat loss, and act as a barrier to protect underlying structures from trauma (Figure 1.1.3.1) [4].

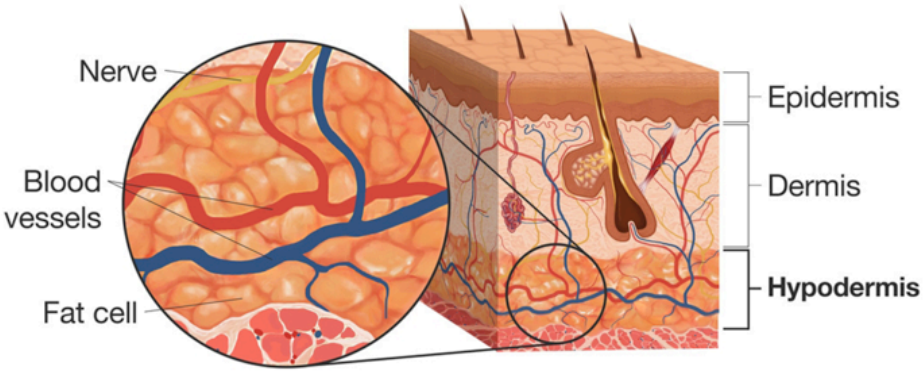

Figure 1.1.3.1 The subcutaneous layer (the hypodermis) [4].

1.2 The Wound and The Wound Types

As the skin continuously exposed to the external and internal effects, it can be easily damaged and lose its crucial functions in the body [5]. A wound can be defined as tissue lose or disruption to the normal anatomical structure and function of the skin causing physical damage, such as burn, trauma, and accident or resulting from diseases, such as cancer, diabetes, hypertension, and rheumatologic and inflammatory disease [6, 7]. Wounds can range from a simple break in the epithelial integrity of the skin or extending into hypodermis with damage to other structures such as tendons, muscles, vessels, nerves, parenchymal organs and even bone. Wounds can be clinically classified as acute and chronic depends on the duration of the healing, which is an essential parameter for wound repair and injury management. Acute wounds are those that can be occurred as a result of surgical operations or traumatic tissue loss and repair themselves completely within the relatively short period of time, usually in 1-4 weeks, with minimal scar. Chronic wounds are more complicated wounds, which are a combination of large tissue loss and infection, cause the result of various parameters, such as burns, arterial and venous insufficiency, pressure, vasculitis, and naturapathic and fail to heal through the normal healing process within a certain time [7].

1.3 The Wound Healing

Wound healing is a complex and continuous process, which is the result of coordinated interactions between various biological and immunological systems such as blood cells, parenchymal cells, extracellular matrix, and soluble mediators, to construct the integrity of the damaged tissue. Wound healing possesses four time-dependent phases: (i) coagulation and hemostasis, (ii) inflammation, (iii) proliferation, and (iv) wound remodeling (Figure 1.3.1) [8-11].

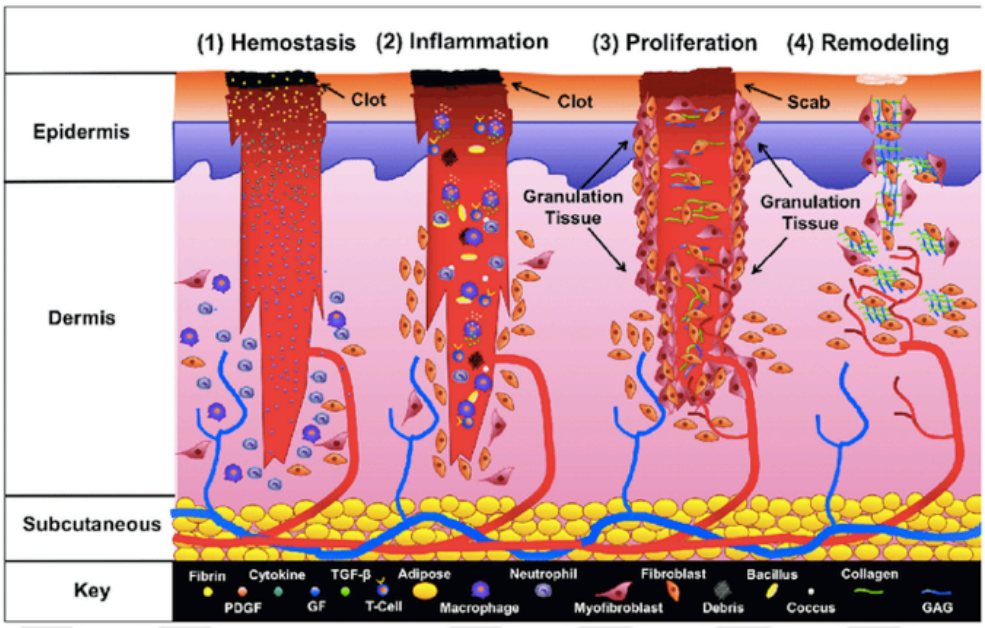

Figure 1.3.1 Stages of Wound Healing [11].

The coagulation and hemostasis phase begins immediately after a wound forms and lasts approximately 1-3 days. The hemostasis is carried out through subsequent occlusion of the larger damaged blood vessels and smooth muscle contraction. The activation and aggregation of platelets and release of clotting factors at the vessel wall injury site triggers the coagulation that supports the thrombosis of the damaged vessels. Platelets construct a clot, and are bound together by fibrin. Several cytokines are released, which recruit leukocytes, to the site of injury to start the inflammation phase [11, 12].

In the inflammation phase, leukocytes, also known as neutrophils, migrate from the surrounding microvasculature and begin removing foreign or necrotic debris, bacteria, and other contaminants from the wound site. T-cells infiltrate the wound and summon macrophages, which release TGF-β and PDGF, to initiate two important aspects of healing angiogenesis and fibroplasia that are essential for providing the metabolic needs of the healing process (Figure 1.3.2) [12, 13].

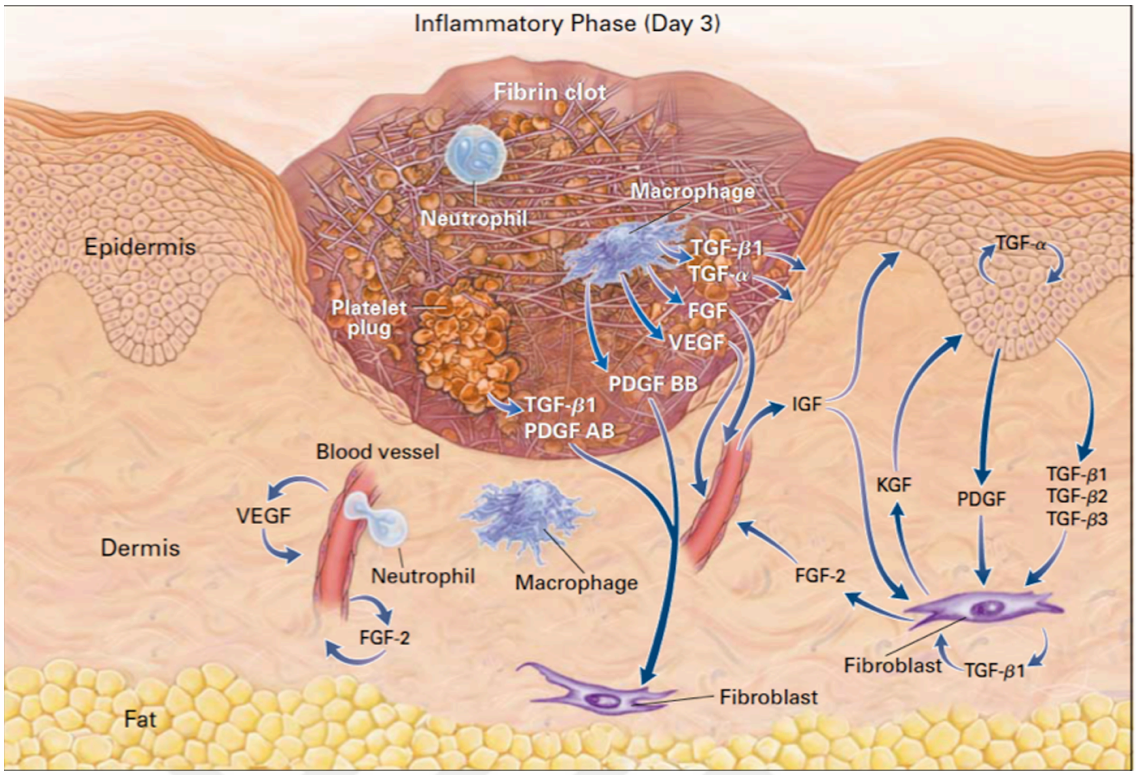

Figure 1.3.2 The inflammation phase [13].

The proliferation phase follows the inflammation phase in the wound healing process. The duration of the proliferation phase is approximately 3 weeks post-injury (3 to 21 days). The macrophages activated already in the inflammation phase start releasing angiogenesis factor (AGF) and fibroblast-stimulating factor, which activate the fibroblasts to produce collagen and glycosaminoglycans (GAGs) and initiates the development of new capillary that supply blood to the wound site. Collagen and the GAGs, along with the neovascularization, structure the granulation tissue that fills in wound defects throughout the proliferation phase. Reestablishing of the wound reduces the size of the defect and brings the wound edges closer together, which occurs in the presence of the myofibroblasts (special fibroblasts) possessing contractile properties between the first and second week after the wound forms. The re-epithelialization process, which involves the migration and proliferation of epithelial cells over the granulation tissue to bridge the defect, takes place in the proliferation phase. This process is essential to wound healing since the new epithelial cells act as a barrier to bacterial invasion and to prevent fluid loss from the wound site. The migration's rate of epithelial cells is greatly affected by the type of surface. A moist surface increases the

migration of epithelial cells, while an eschar covered or dry wound surface prevents the migration to the wound site (Figure 1.3.3) [12, 13].

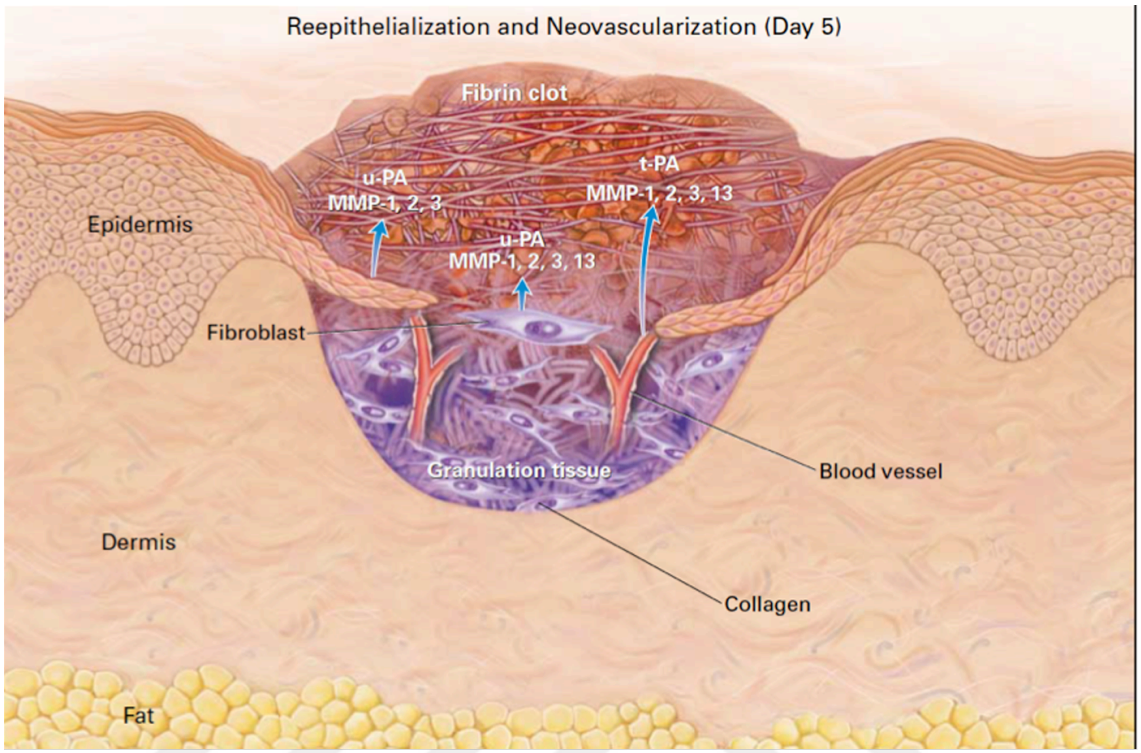

Figure 1.3.3 Re-epithelialization and Neovascularization in Proliferation Phase [13].

Wound healing concludes with the final phase called the remodeling or maturation phase, which begins at approximately 21 days after the injury occurs and lasts at least 1 to 2 years. Collagenases produced during this phase are responsible for the remodeling of the defected wound tissue. The amount of collagen synthesis determines the ultimate strength of the repaired wound. At the end of the remodeling phase, maximal tensile strength is achieved and is almost 80% of the strength of the healthy tissue [12, 13].

1.4 Wound Dressings

There are various local and systemic factors, which affect the wound healing process, such as age, pain, infection, hypothermia, radiation, adequate blood supply and tissue perfusion, nutrition, altered immune function, and an ideal wound dressing. A suitable wound dressing must be chosen based on the wound type for fast wound healing. An ideal wound dressing should be i) biocompatible, non-toxic and non-

allergic, ii) non-adherent to the wound and easy to remove after healing, iii) a good barrier against the bacterial infection and dehydration, and should have ability to iv) provide moist wound environment, v) remove the excess exudate from wound site, vi) allow gaseous exchange between wound site and environment, vii) enhance the cell migration and proliferation, viii) maintain appropriate tissue temperature to improve the blood flow to the wound site, and ix) avoid the scar formation [14-16].

1.5 The Types of Wound Dressings

1.5.1 Conventional wound dressings

The conventional wound care products such as plasters, bandages, cotton wool, tulle, and gauze, are only used for covering to wound and preventing the wound site from external effects. Some sterile gauze dressings fabricated from woven and non-woven fibers of rayon, polyesters, or cotton provide some protection against microbial attacks and can be used for removing excess exudates from the wound site. However, these products need to be changed frequently to prevent the maceration of healthy tissues. Due to the gauze dressings are dry products, they become moistened and adherent because of the exudate of the wound, which causes pain when removing. Bandages produced from cellulose and natural cotton wool or synthetic bandages manufactured from synthetic polymers like polyamide possess different functions, such as retention of light dressings and compression in case of venous ulcers. Besides, conventional skin substitutes such as autografts, allografts, and xenografts have not provided an effective treatment of skin tissue lost due to their drawbacks such as the limitation of donor sites, enzymatic resistance, reduced longevity, and antigenicity. Thus, in recent years, scientists have focused on modern and therapeutic wound dressings with a capacity to accelerating wound healing and preventing bacterial growth together [6-7].

1.5.2 Modern Wound Dressings

Modern wound dressings, which are classified as interactive, bioactive, and passive, are produced not only to cover the wound, but also to improve the wound

healing process. Passive wound dressings are non-occlusive, such as tulle and gauze dressings, used to cover the wound to restore its function underneath. Interactive dressings available in the forms of films, foam, hydrogel, and hydrocolloids can be semi-occlusive or occlusive, which act as a barrier against the microbial attack to the wound site. Based on the type and cause of the wound, various products are available in the market commercially that makes choosing the right wound dressing a challenging task [6-7].

1.5.2.1. Semi-permeable film dressings

Semi-permeable film dressings are manufactured from adherent and transparent polyurethane that allows gas exchange and water from the wound site and provides protection against external bacterial attack, and autolytic debridement of eschar. Prime examples of these films were fabricated occlusive from nylon derivatives possessing an adhesive polyethylene frame. Due to their limited absorption capacity, nylon derived film dressings caused maceration between the wound and the healthy environmental tissue and were not utilized for the wounds having excess exudate and. However, these dressings have significant elastic properties, which makes them highly flexible and to form to any shape without requiring additional tapping. As they are transparent, the patients or physicians can observe the wound closure without removing the film dressings. Thus, semi-permeable film dressings, such as Biooclusive™, Opsite™, Tegaderm™ commercially available in the market, which differ in terms of their adhesive characteristics, vapor permeability, extensibility, and conformability, are suggested for superficial wound, epithelializing wound, and wound with low exudates [6-7].

1.5.2.2. Semi-permeable foam dressings

These dressings are fabricated from hydrophilic and hydrophobic polyurethane foams possessing adhesive borders. The hydrophobic properties of the foams protect the wound site from the liquid, however, allow water vapor and gaseous exchange, which make them suitable dressings for lower leg ulcers and moderate to wounds with excess exudate. Foam dressings, classified adhesive and non-adhesive, can absorb varying quantities of wound exudate based on the wound thickness. Due to their significant

water vapor permeability and absorbance capacity, foam dressings can be used as primary dressings without requiring secondary dressings. However, they require frequent dressings and are not suitable for dry wounds and wounds with lower exudate. Allevyn™, Tielle™, and Lyofoam™ are commercially available foam dressings products in the market [6-7].

1.5.2.3. Hydrogels Dressings

Hydrogels are insoluble hydrophilic materials capacity obtained from both natural and synthetic polymers such as chitosan, alginate, gelatin, collagen, silk, poly(lactic-co-glycolic acid), poly (methacrylates) and polyvinyl pyrrolidine with excellent absorption capacity (70-90 % of its mass). The absorption capacity of hydrogels supports epithelium and granulation tissues in a moist environment, whereas the soft elastic property of hydrogels ensures easy removal from the wound site without any damage. Beside, hydrogels provide a cooling effect to decrease the temperature of cutaneous wounds. Due to their significant properties, such as being non-irritant, non-reactive with biological tissue, and permeable to metabolites, hydrogels are suitable for all stages of wound healing but infected and substantial drainage wounds and can be used for dry necrotic wounds, chronic wounds, ulcers and burn wounds. However, hydrogels have some disadvantages, such as maceration and bacterial proliferation, because of exudate accumulation, which produces stink on the wound site and low mechanical strength, which causes manufacturing difficulties. Some commercial available hydrogels product in the market are Nu-gel™, Granugel™, Intrasite™, Aquaform™, and Vigilon™ [6-7].

1.5.2.4 Hydrocolloid dressings

Hydrocolloid dressings, which are composed of two layers, outer waterproof layer and inner colloidal layer, are one of the most widely used interactive dressings. These dressings are the combination of gel forming agents, such as pectin, gelatin and carboxymethylcellulose, with other materials such as adhesives and elastomers and can be fabricated in the form of foam, sheet and thin film. Hydrocolloids allow water vapor transfer, yet prevent the bacterial attack to wound site. Besides, they have ability to remove debris, absorb wound exudates, and control pH of the wound. These dressings can be used for traumatic wounds and minor burn wounds with moderate exudate

content, as well as for pediatric wound care management, because of their pain free property on removal. Contacting the wound exudate, hydrocolloids construct gel form and ensure moist environment, which helps protection of granulation tissue by absorbing and detaining exudate. Note that, hydrogels are chemically inert to the wound exudate compared to the hydrocolloids. There are some commercial available hydrocolloids in the form of thin films and sheets, such as Duoderm™, Tegasorb™, Granuflex™, Comfeel Contour™. They are mostly utilized as secondary dressings and are not recommended for the wounds possessing high exudate content and neuropathic ulcers [6-7].

1.5.2.5 Alginate dressings

Alginates are mainly derived from seaweed. Alginate dressings in the form of the sheet, ribbon, or rope are obtained from the calcium and sodium salts possessing guluronic acid and mannuronic units. Some studies in the literature have shown that alginates limit the migration of keratinocytes on the wound site. However, it has also been approved that alginates improve wound healing via activating macrophages to produce TNF-α, which starts the inflammatory signals. Applying alginate dressing to the wound, the ions in alginate exchange with blood, which provides a protective film. The usage of alginate dressings is recommended, especially for moderate to massive drainage wounds. They are not suitable for severe wounds with exposed bone, third-degree burn wounds, and dry wounds. As alginate dressings could dehydrate the wound, which causes a long period of healing time, they require secondary dressings. Commercially available alginate dressing products in the market are AlgiDERM™, Maxorb™ Kaltostat™, Sorbsan™, Algisite™, Algosteril™, Tegagen™ [6-7].

1.5.3 Bioactive wound dressings

Bioactive dressings are polymeric biomaterials with biocompatibility, biodegradability, and non-toxic nature and manufactured generally from natural tissues or artificial sources such as collagen, hyaluronic acid, chitosan, alginate, and elastin. Based on the type of wounds, polymers of these materials can be used alone or in combination thereof. These biological dressings can be incorporated with antimicrobials

agents and growth factors to enhance the wound healing process. Due to accelerating endothelial migration upon the wound site and initiating fibroblast formation, which a crucial step in the natural wound healing process, collagen is the most investigated natural polymer as a bioactive wound dressing. Johnson & Johnson, Royce Medical, Promogram™, and Puraply™, are commercially available collagen-based dressings containing a variety of agents in the form of sheet, gel, sheet, sponge, or lattice. Hyaluronic acid (HA), which is a glycosaminoglycan component of the extracellular matrix (ECM) with biodegradable, biocompatible, and lack immunogenicity nature, and chitosan, which promotes the formation of granulation tissue during the proliferative stage of wound healing have also been widely studied as bioactive wound dressings [6-7].

1.5.4 Medicated dressings

Medicated dressings containing growth factors, enzymes, and antimicrobial agents play an essential role in wound healing directly or indirectly by the removal of necrotic tissues and preventing microbial infection. The cellular activities driven by the growth factors in our body control the normal tissue reconstruction process, as mentioned earlier. In the case of chronic wounds, cells and growth factors are incorporated in the wound bed within the clots, which affects the healing process. In the literature, numerous studies have reported the benefits of growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and autologous platelet thrombin, loaded into the wound dressings on the healing process in terms of attachment, proliferation, and differentiation of cells and increasing angiogenesis. Enzymatic removal of death tissues without harming healthy tissue is also one of the crucial steps in the wound healing process. Collagenase and papain based ointments are currently used to remove necrotic tissue. Papain attacks cysteine residue and associated with inflammatory response, whereas collagenase attacks native collagen by the gradual breakdown of tissue. Debridace™ is a commercially available dressing, which increases proteolytic action. Cutisorb™ is a commercially available antimicrobial dressing in the market. Fibrous hydrocolloid, silicone gels, and polyurethane foam and films containing silver and antiseptic iodine loaded membranes are also investigated as

antibacterial wound dressings to prevent or combat infections especially for diabetic foot ulcers [6-7].

1.5.5 Composite dressings

Composite dressings, complex products possessing multiple layers, are suitable for both partial and full-thickness wounds, untreated infected wounds, the third-degree burns, and moderate to massive drainage wounds as either a primary or secondary dressing. Most of the composite dressings possess three layers: i) an outer layer, which protects the wound from external factors and infection, ii) a middle layer made of absorptive material, which retains moisture environment and assists autolytic debridement, and iii) a bottom layer obtained from non-adherent material, which prevents from sticking to young granulating tissues. Composite dressings are more expensive compared to the other wound dressings and may also possess an adhesive border of transparent film or non-woven fabric.

1.5.6 Tissue-engineered skin substitutes

Over the three decades, the attraction on tissue engineering (TE) field, which explores the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ by applying the principle of engineering and life sciences, has been increasing significantly [17]. The general principles of TE involve combining living cells and signaling molecules with a natural/synthetic scaffold designed to mimic the structure of a particular tissue [18]. The approach of skin tissue engineering consists of three essential steps: i) increasing the number of healthy skin cells in the laboratory, ii) seeding them on a scaffold, and iii) applying this cell-scaffold structure for promoting wound healing [19].

Tissue-engineered skin substitutes are developed in three-dimensional structure as the natural extracellular matrix (ECM) to host cell adhesion, growth, and differentiation to obtain structural and fully functional skin tissue. The three-dimensional scaffolds can not only cover wound and provide a barrier against external microbial attacks as wound dressing, but also can support adhesion and proliferation of both keratinocytes and dermal fibroblasts for skin tissue engineering [20]. An ideal scaffold should exhibit

suitable mechanical and physical characteristics and possess appropriate surface chemistry, nano and microstructures to ensure cellular attachment, proliferation, and differentiation. Even though many significant developments have been achieved in the past decades, recently available skin tissue substitutes often suffer from different problems, such as scar formation, poor integration with host tissue, and wound contraction [21].

AlloDerm™ (LifeCell Corporation), a naturally derived dermis fabricated by using lyophilization technique after decellularization treatment, has a fibrillar and porous structure, which mimics the natural structure of human skin (Figure 1.5.6.1 a, b). Due to its human origin, AlloDerm™ has been utilized in the reconstruction of burn wounds as an autograft. Integra™ (Integra Life Sciences), another acellular natural product, was prepared by using a porous composite of bovine collagen-chondroitin-6-sulfate possessing an outer silicone covering. The outer silicone layer, which is removed 2-3 weeks after the application on the defected wound tissue upon the vascularization of the dermis, acts as a temporary barrier preventing bacterial attacks and water loss (Figure 1.5.6.1 c, d) [22]. The porous collagen layers of Integra™ were fabricated by lyophilization of dispersion of collagen to act as a support structure for capillary growth and host fibroblast infiltration. Due to donor site limitations, Integra™ has been widely used as wound closure for severe burn wounds. Another vital product available in the market traded as Apligraf™ is an FDA approved skin substitute made of fibroblasts and keratinocytes containing type I collagen for chronic venous leg ulcers and diabetic foot ulcers. Due to all components of Apligraf™ are very similar to human skin, the vascularization, and well integration into the wound bed promote the wound healing within 14 days (Figure 1.5.6.2 a, b). Other few substitutes are Orcel™, Laserskin™, Biobrane™, Bioseed™, and Hyalograft3-DTM [22].

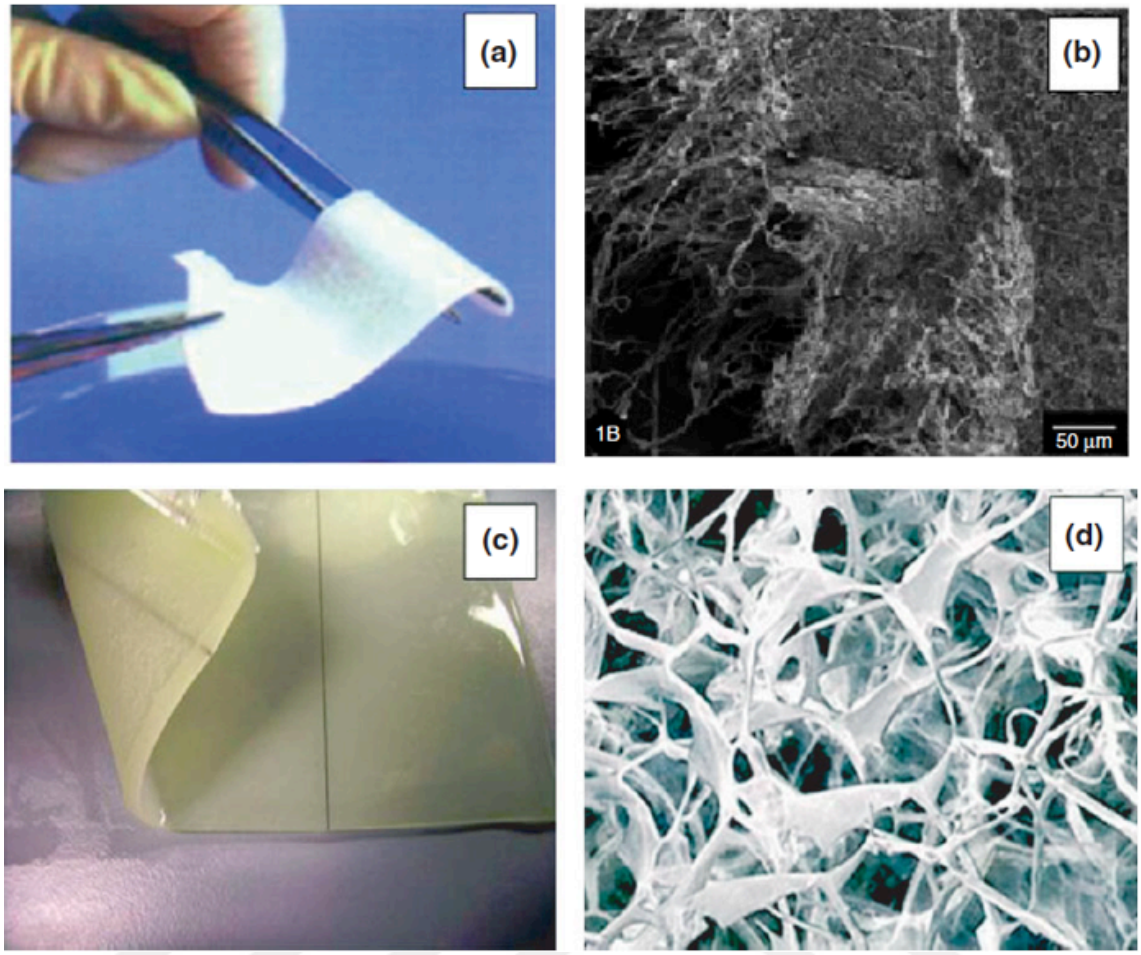

Figure 1.5.6.1 Acellular skin substitutes: (a) appearance of Alloderm™ native tissue, (b) SEM micrograph of the surface, (c) Integra™ template with silicon layer in situ, and (D) Porous sponge-like collagen–glycosaminoglycans structure of Integra™ [22].

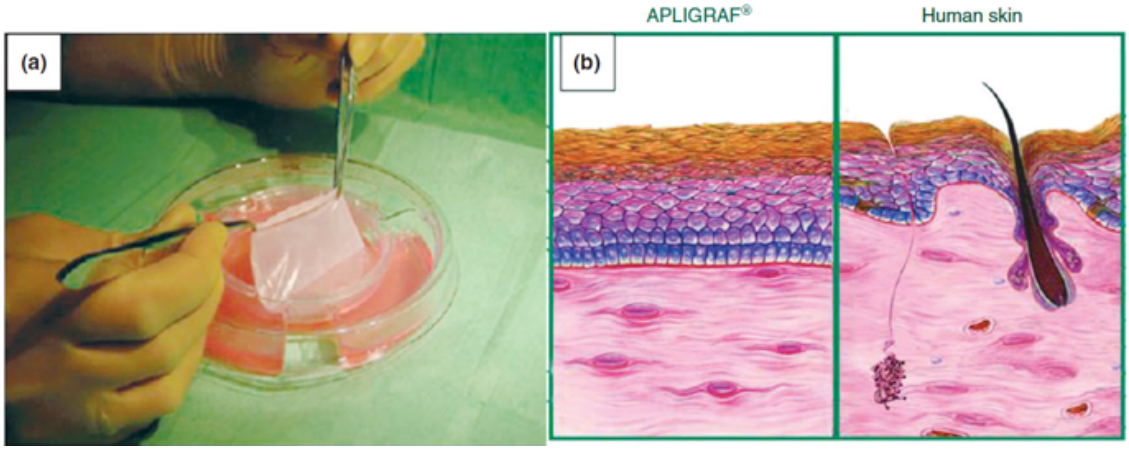

Figure 1.5.6.2 Bilayered skin substitutes: (a) Apligraf™. (b) Comparison of Apligraf™ and natural human skin; Apligraf™ exhibits similar structure as the natural skin [22].

1.6 Wound Dressing Preparation Methods

The wound dressings are mostly fabricated in the forms of film, foams, sponges, or porous scaffolds by using different methods, such as phase separation, gas foaming, solvent casting, and electrospinning [23].

Phase separation is one of the most common techniques used to fabricate porous skin substitute, in which the solution temperature is lowered to induce the phase separation of the homogeneous polymer solution with an appropriate concentration. In this method, the temperature is decreased until freezing the solution to obtain a solid-liquid de-mixing solution, which forms frozen solvent and concentrated polymer phases. Following that, the solvent crystals present in the de-mixing solution are removed through to obtain a porous scaffold. The pore size and the interconnectivity can be arranged by changing the various thermodynamic and kinetic parameters, such as polymer concentration, types of polymer, rate and time of freezing, and the strategy of thermal quenching strategy [24].

Solvent casting, in combination with particulate leaching, is another widely used method for the preparation of porous scaffolds. In this method, firstly, a polymer is dissolved in a proper solvent. A predetermined-sized porogen agent, such as sugar, salt, or similar agent, is then added in the polymer solution. The mixture of polymer/porogen/solvent is transferred into a mold having desired geometry for solvent evaporation. Alternatively, the polymer solution can be directly poured into the mold filled with a specific-sized porogen agent. After evaporation of the solvent, the polymer/porogen composite material is dissolved in a suitable solvent to remove the porogen agent; thus, a porous scaffold can be obtained. Solvent casting is not only a versatile and straightforward method, but also it provides efficient control over the pore size and geometry by adjusting the composition of the porogen, shape, and dimension [24].

1.7 Electrospinning

Electrospinning is a versatile and straightforward technique to produce fibers with diameters in the range of nanometers to micrometers by applying electrostatic forces [25, 26]. The basic electrospinning device comprises a high voltage power supply, which transmits the charge to the polymer solution at predetermined flow rates, a digital

syringe pump that forces the solution through a capillary tube, and a grounded conductive collector. In the electrospinning process, an electric field inducing a charge on the liquid surface is applied to a polymer solution, which is held by its surface tension at the end of a capillary tube (Figure 1.7.1). A force directly opposite to the surface tension is occurred by repulsion of mutual charge. The increasing intensity of the electric field triggers the surface of the solution to form a conical shape known as the Taylor cone. The repulsive electric force overcomes the surface tension at the critical value of the electric field, which causes a jet stream of the solution from the tip of the Taylor cone to the collector. The solvent evaporates during the travel of jet in the air, and polymer fibers are deposited randomly on the collector to construct a non-woven fabric. Some parameters affect the electrospinning process including solution properties, such as conductivity, surface, and tension viscosity, controlled variables, such as the applied voltage, flow rate, distance between the tip and the collector, geometry of collector, and needle gauge, and ambient parameters such as humidity, air velocity and temperature in the electrospinning chamber [27].

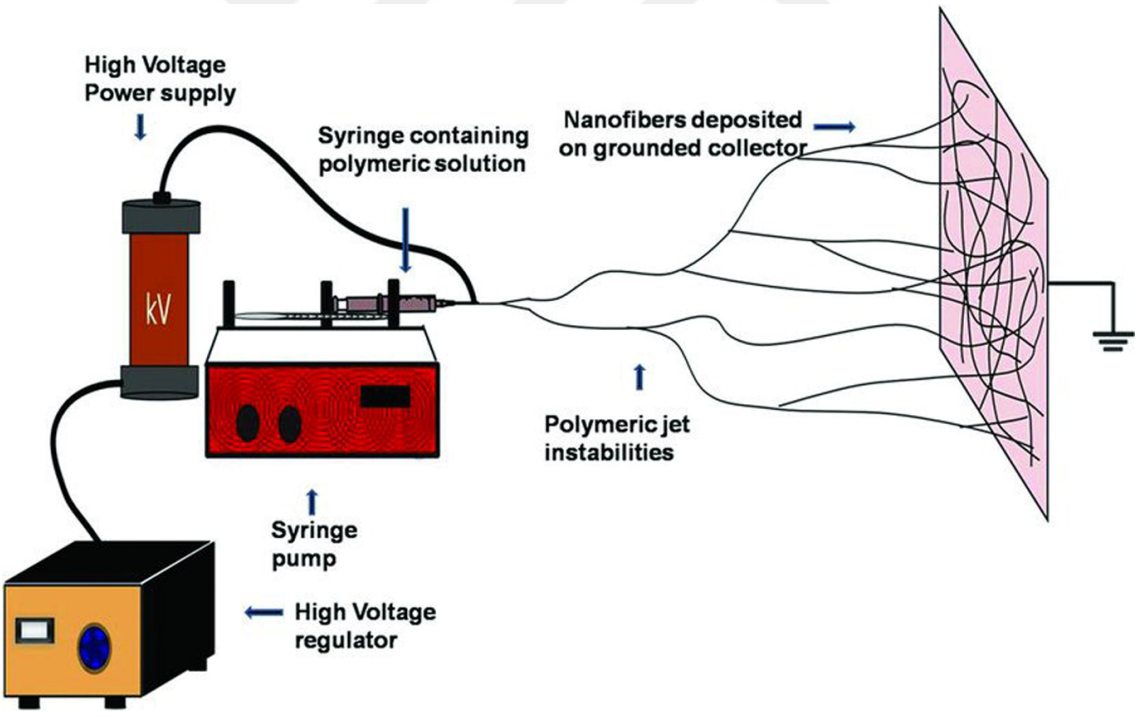

Figure 1.7.1 Schematic representation of electrospinning apparatus [30].

Due to their unique properties, such as surface-area-to-volume, interconnected pore structure, adequate mechanical strength, gas permeability, promoting cell migration and adhesion, moisture retention, and highly efficient absorption capacity,

electrospun nanofibers have been applied in various biomedical fields as three-dimensional scaffolds for tissue regeneration of bone, cartilage, tendon, dental, blood vessels, muscle, heart valves, muscle, neural tissue, and skin (Figure 1.7.2) [30-32].

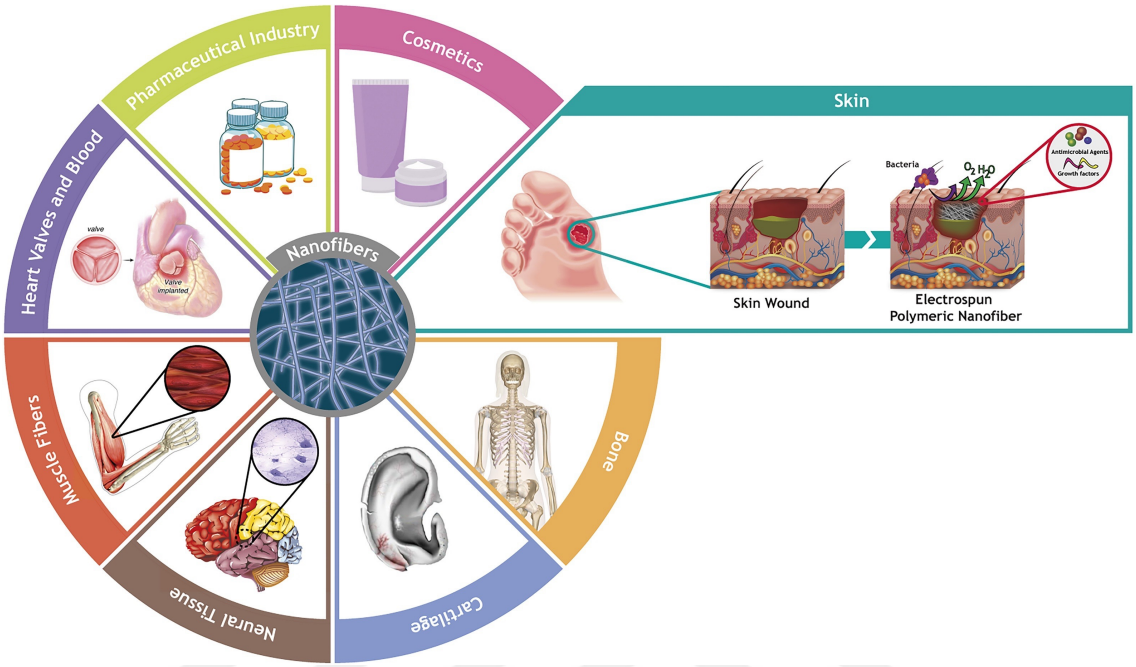

Figure 1.7.2 Illustration of applications of electrospun nanofibers in different biomedical fields [31].

1.7.1 Polymeric Electrospun Nanofibers for Wound Dressing

The electrospun membranes can be prepared from a variety of biodegradable and biocompatible, both synthetic and natural polymers and their blends. Figure 1.7.1.1 shows the scanning electron micrographs of various polymeric nanofibers investigated for applications in wound healing applications [30-32].

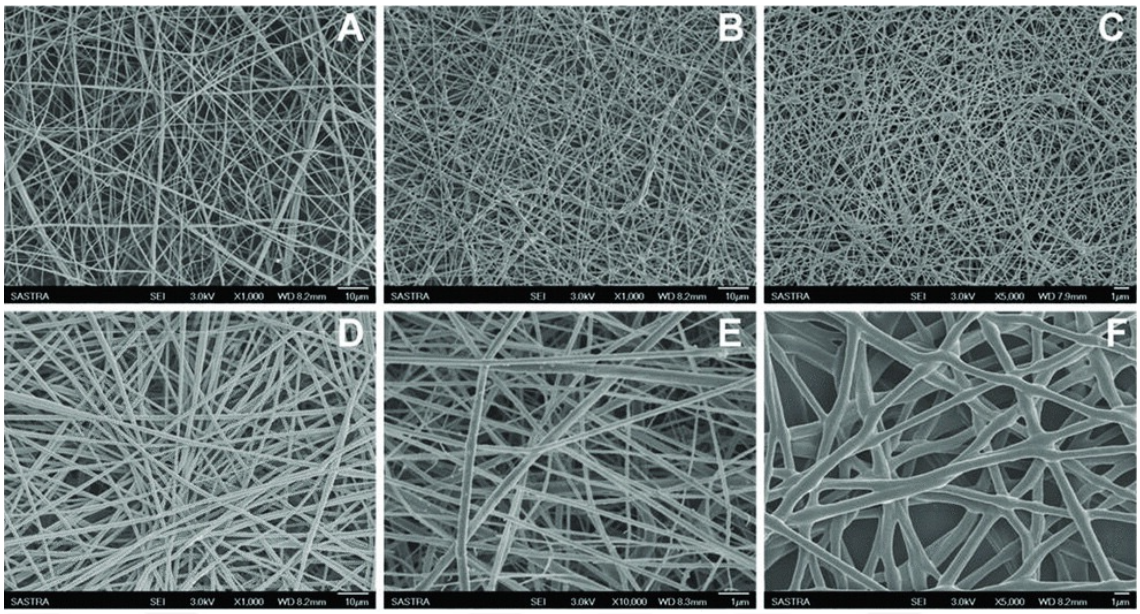

Figure 1.7.1.1 Different types of polymeric nanofibrous scaffolds investigated for skin tissue engineering applications. A) Poly(1,4 butylene succinate) (PBSu); B) PHBV-PBSu; C) Chitosan-PVA; D) PLGA; E) PLA; F) PHBV [30].

1.7.2 Natural Polymers

Due to their biological characteristics, biocompatibility, similarity to macromolecules recognized by cells, and biodegradability, various natural polymers, such as collagen, fibrinogen, chitin, chitosan, some glycosaminoglycans, starch, dextran, alginate and microbial polyesters (polyhydroxyalkanoates (PHA)), have been widely used in biomedical applications. Nanofibrous scaffolds prepared from natural polymers not only can mimic the native ECM but also have the structural similarities. There are numerous reports on the application of nanofibrous scaffolds made of natural polymers for skin substitutes and wound dressings [30-32].

Collagen, the most abundant protein in the body, is the main component of the native ECM with a fibrillar structure possessing a diameter in the range of 50–500 nm that provides tensile strength and structural integrity to tissues. Besides, the adhesion parts in collagen ensure significant surface chemistry for cell attachment. Thus, numerous studies investigated nanofibrous skin substitutes and wound dressings prepared from collagen and reported the significant efficiency of them on the attachment, adhesion, and proliferation of the cells actively involved in the wound healing process [30-32].

Gelatin is a biodegradable and biocompatible natural polymer and is derived from natural collagen existing in the tendon and ligaments by partial hydrolysis, in which the collagen's triple-helical structure is turned into single-strand molecules. Some studies have shown the promoting effects of gelatin on cell adhesion and migration due to its arginine-glycine aspartic acid (Arg-Gly-Asp, RGD) sequences that enable recognition of the integrin protein on the cell surface, and lower immunogenicity. Nanofibrous scaffolds prepared from gelatin by using electrospinning with an interfiber distance in the range of 5–10 μm are good candidates for skin substitute since the organized layers of the scaffolds are similar to the native skin tissue. Electrospun gelatin nanofibrous scaffolds cross-linked by glutaraldehyde vapor with strengthened mechanical characteristics enhance the proliferation of human dermal fibroblasts as an appropriate wound dressing. Silver nanoparticle loaded electrospun gelatin membranes have been studied as wound dressing materials to show their antibacterial activity against the gram-negative and gram-positive bacteria [30-32].

Chitin, chitosan, silk, fibrinogen, and cellulose have been utilized to prepare electrospun nanofibrous wound dressings. Chitosan is derived from deacetylated chitin, which mainly exists in shells of crustaceans and arthropod exoskeletons. Due to their antifungal, antibacterial, and haemostatic characteristics, chitin and chitosan have been extensively investigated as a dressing material for wound and burn applications. Besides, it has been shown that chitosan can activate the macrophages and production of cytokine, as well as provide fibroblast proliferation. Chitosan can also trigger a vast amount of natural hyaluronic acid synthesis at the wound site that enhances wound healing and decreases scar formation [30-32].

Silk proteins, a typical fibrous protein produced mainly by silkworms, are found as hydrophilic sericin and hydrophobic fibroin. Fibroin is a biocompatibility and biodegradability polymer possessing lower immunogenicity and gas permeability properties, which make it a promoting candidate for wound healing applications. Thus, electrospun silk fibroin scaffolds have attracted some attention as wound dressings, recently. Min et al. investigated three different silk fibroin (SF) matrices, a matrix of SF film, a woven matrix from SF microfibers, and a non-woven matrix of SF nanofibers, to observe promotion capacity on the cell adhesion and proliferation and shown the better spreading of the coated collagen on SF nanofibrous matrix [30-32].

Fibrinogen is a glycoprotein synthesized by the liver and plays a crucial role in the coagulation and wound healing process. Wnek et al. fabricated electrospun fibrinogen

nanofibers for the first time in the literature. Since fibrinogen is not soluble in HFIP alone, they used a mix solution prepared from HFIP, low amount of Earle's salts (minimum essential medium, ME), human, or bovine fibrinogen fraction I. However, it has resulted that fibrinogen nanofibers lose their integrity *in vitro* cell culture in few days. Thus, Sell et al. evaluated three types of chemical cross-linkers: EDC, genipin, and GA vapor, to improve the mechanical properties of fibrinogen. According to their results, EDC and genipin not only improved the mechanical properties of the electrospun scaffolds but also decreased the degradation rate, whereas Glutaraldehyde cross-linking could not improve the mechanical features of fibrinogen [33].

Hyaluronic acid belongs to glycosaminoglycans (GAGs) family and is one of the significant components of the native ECM. Hyaluronic acid has essential functions in the healing process, such as angiogenesis by enhancing the mitosis of epithelial cells, adjusting phagocytosis by regulating the movement of the macrophages, which promotes wound healing and decreased scar formation. Some studies about electrospun HA mats have been conducted in the literature and reported difficulties about its electrospinnibility due to its water capacity, surface tension, and high viscosity [30-32].

1.7.2.1. Poly(3-hydroxybutyrate-co-3-hydroxy valerate) (PHBV)

Poly(3-hydroxybutyrate-co-3-hydroxy valerate) (PHBV), a natural polymer and member of polyhydroxyalkanoates (PHA), serves significant to healthcare society as wound healing material [34]. Kuppan et al. have shown the suitability of PHBV nanofibers on the adhesion and proliferation of human skin fibroblasts. In another study that belongs to this group reported the biocompatibility of PHBV nanofibers for keratinocytes adhesion and proliferation. Peripheral blood lymphocytes cultured on PHBV showed that genes related to the activation of lymphocytes were down-regulated after 2 and 3 days of culture. These results approve the conformity of PHBV electrospun nanofibers for skin tissue engineering [35].

1.7.3 Synthetic polymers

Synthetic polymers, such as poly(α-hydroxy acids), poly(ε-caprolactone), poly(α-amino acids), poly(orthoesters), poly(anhydrides), poly(alkyl-2-cyanoacrylates), and

poly(phosphazenes) have been used for preparing electrospun membranes as potential wound healing materials due to their significant properties including excellent easily electrospinnibility, mechanical strength, thermal stability, minimal inflammatory response and adjustable degradation time *in vivo* and *in vitro* [36].

Khil et al. manufactured electrospun nanofibrous Polyurethane (PU) membranes as a scaffold for the first time in the literature. According to their results, PU membranes exhibited significant oxygen permeability and fluid drainage ability. The *in vitro* cell studies of the membranes approved their biocompatibility and antibacterial activity. In addition, animal studies revealed that PU membranes displayed a well-organized dermis and granulation tissue after 15 days of treatment [37].

1.7.3.1. Poly(D, L-lactide-co-glycolide) (PLGA)

Among the poly(α-hydroxy acids), poly(D, L-lactide-co-glycolide) (PLGA), FDA approved biocompatible copolymer, has been widely used in wound healing applications, due to its unique properties such as easily electrospinnibility, minimal inflammatory response and adjustable degradation time *in vivo* and *in vitro* [38-40]. Blackwood et al. electrospun poly(lactic acid-co-glycolic acid) (PLGA) and PGA different ratios to obtain nanofibrous dermal substitutes, and investigated in terms of biodegradability *in vivo* and *in vitro*. Their results revealed that increasing the percentage of glycolide to lactide in the copolymer increases the rate of degradation both *in vivo* and *in vitro* [41]. Cui et al. blended poly(ethylene glycol) (PEG) with poly(DL-lactide) (PDLLA) to fabricate electrospun nanofibers with more hydrophilic surface and bulk degradation behaviour [42]. In another study, Kumbar et al. assessed electrospun PLGA nanofibers in terms of its morphology, the correlation between fibroblast proliferation and diameters of PLGA nanofibers, and demonstrated excellent proliferation of fibroblast seeded on the scaffolds with fiber diameter in the range of 350–1000 nm [43].

1.7.4 Natural/Synthetic Blend Polymers

The recent studies have revealed that the wound dressing materials prepared by electrospun polymer blends of synthetic and natural polymers demonstrate the

synergetic effect and desirable advantages on the wound healing process, which cannot be achieved by individual polymers. Shi et al. prepared electrospun quaternary ammonium salts-functionalized PCL-gelatin hybrid membrane for a novel wound dressing possessing long-lasting antibacterial properties [44]. In another study, Tra Thanh et al. fabricated plasma-treated electrospun PCL scaffold coated with silver nanoparticles (AgNPs) embedded in gelatin to enhance its antibacterial property and minimize the adhesion of scaffold on the wound area [45]. In 2018, Liao et al. developed a hybrid alginate hydrogel crosslinked by calcium gluconate crystals deposited in PCL-b-PEG-b-PCL porous microspheres for wound healing [46]. Ehterami et al. prepared electrospun PCL/collagen nanofibrous matrices loaded with insulin delivering chitosan nanoparticles as a potential wound healing material [47]. In another study conducted by Paskiabi et al., an electrospun PCL/gelatin fibers containing terbinafine hydrochloride as an antifungal wound dressing were provided [48]. Abdalkarim et al. prepared electrospun PHBV/cellulose reinforced nanofibrous membranes with ZnO nanocrystals for wound healing [49]. In 2015, Lei et al. prepared PHBV/silk fibroin nanofibrous scaffolds for repairing skin tissue [50]. Varshosaz et al. investigated electrospun poly(methyl vinyl ether-co-maleic acid)/PLGA nanofibers loaded with montelukast as a new wound care material [51]. In another study, Liu et al. provided ciprofloxacin-loaded PLGA/sodium alginate electrospun mats for wound healing [52]. Daranarong et al. prepared electrospun fibrous scaffolds of polyhydroxybutyrate (PHB) with various loadings of poly(L-lactide-co-ε-caprolactone) (PLCL) for nerve tissue engineering. They showed that PLCL/PHB blend scaffolds had reduced tensile strength and better adhesion and proliferation performance of olfactory ensheathing cells (OECs) compare to neat PLCL electrospun scaffolds [53]. In another study, Ding et al. fabricated fibrous mats of PHB/PCL/58s blends using electrospinning and sol-gel methods and investigated the cell adhesion, viability, proliferation and ALP activity of MG-63 osteoblast-like cells on the scaffolds [54]. Ublekov et al. provided electrospun poly(β-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)/organo-modified montmorillonite (OMMT) nanocomposite fibrous scaffolds and reported the mechanical properties of the hybrid scaffolds [55].

1.7.5 *Centella Asiatica* (CA)

One of the essential characteristics of an ideal wound dressing is acting as a barrier against the microbial attacks and protects the wound area from infection, as mentioned earlier. Thus, preventing infection on the wound site, some antibacterial and antimicrobial agents, as well as natural medical plant extracts, have been incorporated into electrospun membranes [56].

Centella Asiatica (CA), a medicinal plant, has been used in traditional medicine in many parts of the world, including Asia and the Middle East countries to treat small-scale wound areas, hypertrophic scars and damaged skin tissue caused by burns [57-59]. The main pharmacologically active components of CA are saponin-containing triterpene acids and their sugar esters, of which Asiatic acid, asiaticoside, and madecassic acid are considered to be the most effective (Figure 1.7.5.1) [60, 61]. Among these components, asiaticoside is the primary therapeutic substance and accelerates the wound healing process and reduces scar formation, while asiaticoside provides fibroblast proliferation and ECM synthesis in the wound area. It was also reported that madecassoside effects significantly on wound healing in terms of antioxidant activity, collagen synthesis, and angiogenesis [62]. Kosalwatna et al. reported that the cream containing 1% CA extract cream exhibits significant wound healing of chronic ulcer in width, length, and depth after 21 days of usage [63]. Shukla et al. investigated the topical application of asiaticoside in regular as well as in diabetic animals and demonstrated a significant enhancement wound healing activity as assessed by an increase in collagen synthesis and tensile strength of the wound tissues [64]. In another study conducted by Maqugart et al., triterpenes from CA were shown to increase the remodeling of the collagen matrix and stimulate glycosaminoglycan synthesis in a rat model [65]. Liu et al. approved antioxidative activity, collagen synthesis, and angiogenesis properties of the madecassoside isolated from CA for burn wound healing in mice model [66]. In another study published in 2016 by Zhang et al., it was developed a porous microsphere for the topical delivery of asiaticoside to increase its absorption and improve its therapeutic effects, as well as to supply a novel preparation with potential for clinical wound treatment [67]. Sikareepaisan et al. investigated the potential of electrospun gelatin fiber mats as carriers for topical delivery of a methanolic crude extract of CA and the release characteristic of asiaticoside from the CA-loaded gelatin fiber mats [68]. In a similar

study belong to Yao et al., it was investigated the potential of electrospun gelatin membranes containing CA extract as topical/transdermal wound dressings [69]. Based on the previous studies that showed the significant healing and scar reducing effects of CA on the different types of wounds, including traumatic, surgical, diabetic, and burn, in the present study, we chose the extract of CA to obtain active and innovative wound dressing material.

On the other hand, the polymers containing quaternary ammonium salts have widely studied to fabricate antimicrobial wound dressings with no toxic effect. Chemically modified poly(4-vinyl pyridine) (poly(4-VP)) is one of these polymers and has been investigated in the literature as wound healing material.

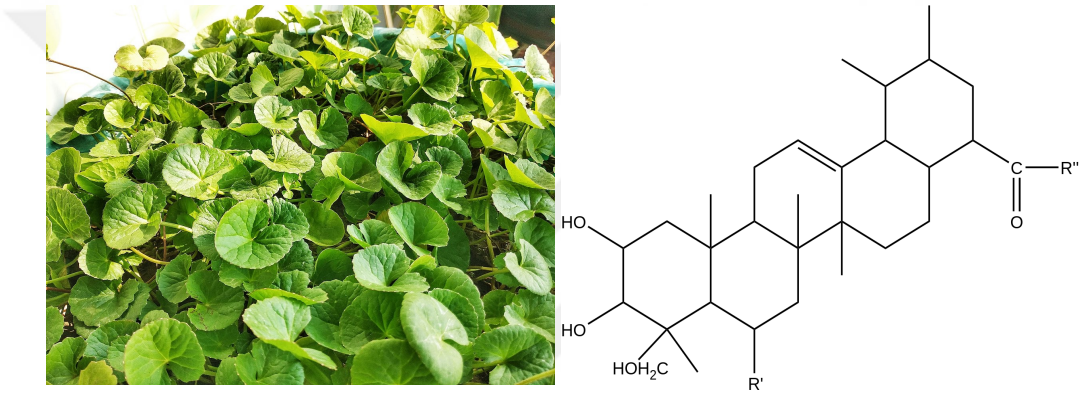

Figure 1.7.5.1 Natural Appearance and Chemical Structure of *Centella Asiatica*

In this study, we aimed to fabricate and characterize a bilayered fibrous wound dressing prepared by electrospinning technique, which consists of CA extract containing electrospun PLGA/PHBV membrane as lower layer contacting directly the wound surface and electrospun quaternized poly(4-VP) as upper layer protecting the wound area from microbial attacks. We also investigated the viability, adhesion, and attachment of human fibroblast cells on the wound dressing.

Chapter 2

2 MATERIALS AND METHODS

2.1 Materials

Poly(D,L-lactide-co-glycolide) (PLGA, DL-Lactide:Glycolide copolymer, ratio M/M%: 75/25, MW=120,000; CAS No.26780-50-7) was purchased from Purac Biomaterials. Poly(3-hydroxybutyrate-co-3-hydroxy valerate) (PHBV, natural origin, PHV content 12 mol%, Mn=280,000, Mw= 690,000; CAS No.80181-31-3) was purchased from Aldrich co (USA). 4-Vinylpyridine (CAS No.100-43-6) was purchased from Aldrich. Centella Asiatica (CA, natural extract in powder form, CAS No.16830-15-2) was obtained from Wuhan Yuancheng Technology Development Co. Ltd. (China). 6-Bromo caproic acid (%97, CAS No. 4224-70-8) was purchased from Aldrich. 2,2'-Azobis(2-methylpropionitrile) (AIBN, CAS No.78-67-1) was purchased from Aldrich. Human fibroblast cells were kindly donated by Erciyes University Genom and Stem Cell Center, Kayseri. All other chemicals and solvents are used without further purification.

2.2 Fabrication of CA Containing Bilayered Wound Dressing

Bilayered wound dressing was fabricated by sequential and continuous electrospinning of quaternized poly(4-vinyl pyridine) (upper layer) on the CA containing electrospun PLGA/PHBV blend membranes (lower layer). All electrospinning experiments were conducted at room temperature, and all membranes were vacuum dried overnight, stored in dry cabinets, and used for further studies. The fabrication process was conducted in two stages:

2.2.1 Preparation of Lower Layer (CA Loaded PLGA/PHBV Electrospun Membranes)

We first optimized the concentrations of PLGA/PHBV blend solutions and determined the type of solvent/solvent mixture for electrospinning in order to obtain electrospun membranes possessing a bead-free structure and uniform fibers with minimum average diameter. The other electrospinning parameters, such as applied voltage, flow rate, and the distance between the tip and the collector, were kept constant. To do so, we prepared PLGA/PHBV blend solutions at the ratio of 75:25 (w/w) with different concentrations (5, 10, 20%, w/v). Chloroform ($CHCl_3$), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and their mixture were used in different ratios (50:50, 75:25 v/v) as the solvent. Each blend solution was magnetically stirred at room temperature for 3 h, filled into a 10 mL syringe with 21G needle (with an inner diameter of 14.53 mm), and then placed on a syringe pump (NE-1000 Syringe Pump, New Era Pump Systems, USA). The electrospinning parameters were set as 20 kV of high voltages (NE100, Inovenso, Turkey), 1.5 ml/h of the flow rate, and 15 cm of the distance between the tip and the collector.

***Centella Asiatica* Loading**

Based on the results above, the PLGA/PHBV blend (75:25 w/w) solution prepared in pure HFIP was used for CA loading experiments. Briefly, PLGA and PHBV pellets were dissolved in HFIP at the ratio of 75:25 (w/w) and CA extract were added to the PLGA/PHBV blend solution at different ratios (1, 5 and 10%, w/v) with the final concentration of 20% w/v. The electrospinning parameters mentioned above were applied the voltage: 20 kV, the flow rate: 1.5 ml/h, and the distance between the tip and the collector: 15 cm.

2.2.2 Preparation of Upper Layer (Electrospun Poly(Q4-VP) Membrane)

Synthesis and Quaternization of Poly(4-VP)

Electrospun quaternized poly(4-VP) membrane that we designed in our previous study was used as the upper layer of the wound dressing [70]. In the mentioned study, we applied 4 and 8 days of quaternization process and achieved antimicrobial

characteristics with 8-days of quaternization. Therefore, we applied the same procedure in our current study to obtain an antimicrobial polymer. Briefly, we first synthesized poly(4-VP) from the 4-vinyl pyridine monomer by free radical polymerization. The AIBN was used as the initiator, and DMF used as the solvent. We kept the monomer/initiator ratio at 500:1 and purged the mixture with nitrogen for 30 min before polymerization. The reaction occurred at 65 ^{o}C for 20 h. Afterward, the product was purified by precipitation from toluene three times and stored in a vacuum oven for the quaternization step. For quaternization, synthesized poly(4-VP) was reacted with 6-bromocaproic acid as a quaternization agent at the ratio of 1:2 (v/v) in methanol, and the mixture was purged with nitrogen gas for 30 min. The solution was refluxed in the oil bath at 65 ^{o}C at 450 rpm for eight days. The quaternized polymer (poly(Q4-VP)) was successfully obtained after the product was purified by precipitation from diethyl ether [70]. Quaternization, which can be accepted as a confirmation for the antimicrobial characteristic based on our previous study, was confirmed by FT-IR analysis. Note that we did not repeat the antimicrobial test here, as we already reported it in İşoğlu et al. [70].

Electrospinning of Poly(Q4-VP)

The quaternized poly(Q4-VP) were dissolved in DMF for preparing electrospinning solutions at different concentrations (40, 50, 60, 70% w/v). Each solution was fed into a 10 mL syringe with a 21G needle (with an inner diameter of 14.53 mm) and seated on a motorized and programmable syringe pump (NE-1000 Syringe Pump, New Era Pump Systems, USA). The electrospinning process parameters were set as follows: the applied voltage was in the range of 18 kV, the distance between the tip and the collector was 15 cm, and the flow rate was 1 ml/h.

2.2.3 Characterization

2.2.3.1. Morphological Study

Scanning electron microscopy (SEM) (Carl Zeiss EVO LS10, Germany) was utilized to indicate the morphology of all electrospun membranes. The Image J software (National Institutes of Health, Bethesda, MD) was used to calculate the average fiber diameters from SEM images.

2.2.3.2. Fourier Transform Infrared (FT-IR) Analysis

The FT-IR spectra of the electrospun membranes and neat CA extract were recorded by using Nicolet 6700 FT-IR Spectrometer (Thermo Scientific, USA) in the range of 400-4000 cm^{-1}.

2.2.3.3. Thermal Analysis

Thermal properties of electrospun membranes were determined by differential scanning calorimetry (DSC) (Perkin Elmer, USA) at a heating rate of 2 $^{o}C\ min^{-1}$ from 0 to 200 ^{o}C.

2.2.3.4. Absorption of Wound Fluid

In order to mimic wound fluid, Pseudo Extra Cellular Fluid (PECF) was used, which was prepared by dissolving sodium chloride (NaCl), potassium chloride (KCl), sodium hydrogen carbonate ($NaHCO_3$) and hydrogen phosphate (K_2HPO_4) in distilled water at a pH value of 8 [46]. The 1x1.5 cm rectangular samples of electrospun PLGA/PHBV (75:25 w/w, %20 w/v) membranes with CA (1% w/w) and without CA, PLGA/PHBV (75:25 w/w, %20 w/v) film prepared by solvent casting method in Petri dishes were first weighed and placed into the 24-well plate filled with PECF, which were kept in an incubator at 37^{o}C for 24 h. The samples were weighed after the excess of the solution was removed by blotting with filter paper. The percentage of absorption capacity of membranes was calculated as follows:

$$\textbf{Absorption ratio (\%)} = (W_s - W_0)/W_0 \times 100 \qquad \textbf{(2.2.3.4.1)}$$

Here W_0 is the weight of the initial dry sample and W_s is the weight of the swollen sample.

2.2.3.5. Mechanical Analysis

The evaluation of tensile strengths and elongation at the break of electrospun membranes was performed by using Autograph AGS-X 10 kN (Shimadzu, Japan) device. The samples were cut into the strips with 60 mm in length, 20 mm in width possessing a thickness of 0.1 mm. Tensile tests were conducted at the strain rates of $1x10^{-1}$ s^{-1} at room temperature.

2.2.3.6. *In vitro* Drug Release Study

Asiaticoside absorbance was monitored to determine the *in vitro* release profile of the CA from the PLGA/PHBV electrospun membrane at 215 nm. First, the calibration curve of the asiaticoside solution was plotted with 0.5, 0.1, 0.05, 0.01, 0.001 mg/ml in water. PLGA/PHBV (75:25 w/w, %20 w/v) electrospun membrane with CA (1% w/w) was placed into a 10 ml PBS solution, and a determined time interval 1 ml sample solution was withdrawn, and a new PBS solution was added to this medium. The absorbance of the withdrawn sample was measured at 215 nm. The cumulative release of CA was calculated using the following equation;

Release (%) = Volume of sample withdrawn (ml) x P(t-1)/Bath volume (v) + Pt release **(2.2.3.6.1)**

Here Pt is percentage release at time t and P(t–1) is percentage release previous to "t"

2.2.3.7. Assessment of Cell Viability

As dermal fibroblasts have a crucial contribution to the wound healing process, we used human fibroblast cells for assessment of the biocompatibility of the electrospun membranes. Human fibroblasts were cultured with 10 mL Dulbecco's minimum essential medium (DMEM, Gibco® Life Technologies, Darmstadt, Germany) with supplements of 4.5 mg ml^{-1} glucose, 10% fetal bovine serum and 1% penicillin and streptomycin in 10 cm^2 tissue culture flasks. The cells were kept in an incubator at 37°C with 5% CO_2. The viability of the cells on the membranes was determined by using the MTS assay (CellTiter 96®, Promega, Madison, Wisconsin, USA). Electrospun PLGA/PHBV (75:25 w/w, 20% w/v) membranes with (1% w/v) and without CA and

electrospun poly(Q-4VP) were cut into the rectangular shape (5x2.5 mm). The samples were soaked in ethanol solution (70% v/v) for 2 h and kept under UV for 1 h [71, 72]. Afterward, the cells were seeded on the samples placed in 96-well plates ($1x10^4$ cells/well). The cells/membrane structures were incubated at 37°C in a humidified incubator of %5 CO_2 for pre-set days. After 1, 3, and 7 days of culture, the electrospun membrane samples were incubated in 20 µl of MTS reagent at 37°C for 4 h. Subsequently, the absorbance of the medium was measured at 490 nm using a 96-well plate reader (Varioskan Lux, Thermo Scientific, USA) [73].

2.2.3.8. Assessment of Cell Proliferation and Attachment

SEM was used for the assessment of cell attachment and proliferation on the electrospun membranes. The cells/membrane structures were prepared, as described in the previous paragraph. After pre-set days (1, 4, and 7 days), the samples were rinsed three times with PBS buffer to remove non-adherent cells and immobilized with glutaraldehyde solution (2.5% (v/v)) at 4 ^oC for 2 h. Then, each sample was dehydrated through a series of gradient ethanol solutions of 30%, 50%, 70%, 90%, and 100% for ten minutes and air-dried for SEM evaluation [74].

Chapter 3

3 RESULTS AND DISCUSSION

3.1 Fabrication of CA Loaded Bilayered Electrospun Wound Dressing

Lower Layer

It is well known from the literature that the electrospun membranes prepared from blends of synthetic and natural polymers have several advantages comparing to individual polymers [47]. Based on this fact, we produced the electrospun membrane made of PLGA/PHBV blend containing CA to prepare the lower layer of the bilayered wound dressing. We first conducted preliminary electrospinning experiments to adjust concentrations of PLGA/PHBV blend solutions and the type of solvent/solvent mixture. The other electrospinning parameters, such as applied voltage, flow rate, and the distance between tip and collector, were kept constant. SEM images of the membranes possessing bead-free structure and continuous fibers with homogenous diameter distribution were used for the optimization of process parameters.

Figure 3.1.1 shows SEM images of electrospun membranes prepared from different solvents at different concentrations. As seen from Figure 3.1.1, no fibers were formed at 5% polymer concentration independent of solvent, while many beads formation was observed at 10% polymer concentration (Figure 3.1.1 a, b, c, d). However, electrospun PLGA/PHBV (75:25, w/w) membranes, which possess appropriate bead-free structure and homogeneously distributed fibers, were obtained from polymer solutions prepared from pure HFIP and HFIP/CHCl3 mixture at 20% concentration (Figure 3.1.1 f, g, and h). As mentioned before, the operating parameters were set 20 kV of the applied voltage and 1.5 ml/h of flow rate and 15 cm of distance between tip and collector. Image J software was used to calculate the mean of fiber diameters by measuring the diameters of randomly selected 50 fibers in the SEM images. The average fiber diameter of electrospun PLGA/PHBV (75:25, w/w) blend

membranes prepared with solvent mixture were calculated 1.314±0.44 mm (in pure $CHCl_3$), 1.728±0.33 mm (in HFIP/$CHCl_3$ mixture, 50:50, v/v), 1.673±0.28 mm (in HFIP/$CHCl_3$ mixture, 75:25, v/v) and 0.929±0.13 mm (in pure HFIP), respectively (Figure 3.1.2).

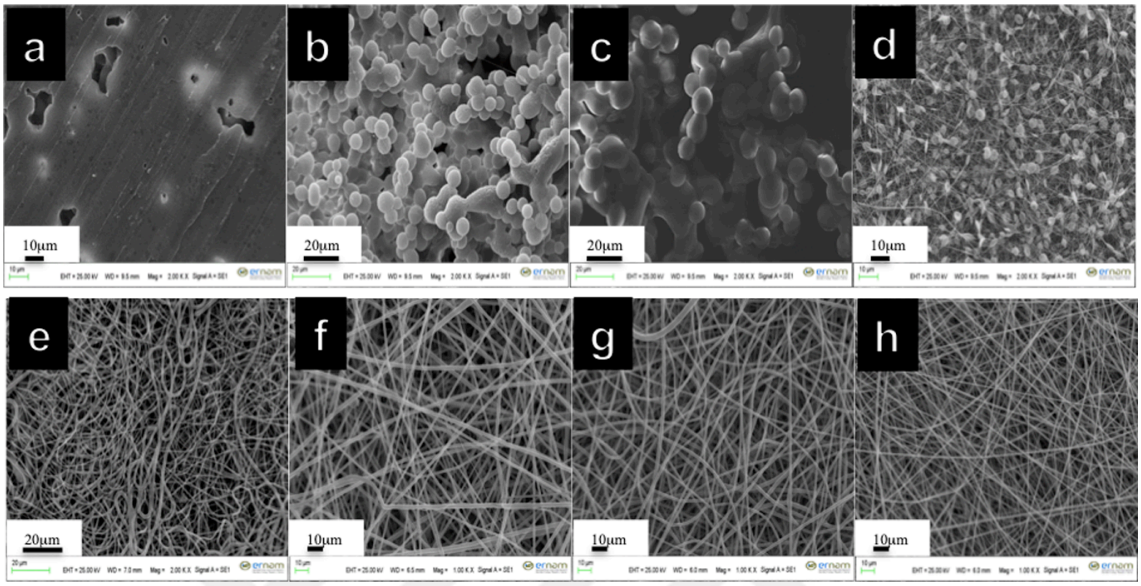

Figure 3.1.1 SEM images of electrospun PLGA/PHBV (75:25, w/w) membranes as a function of concentrations and type of solvent/solvent mixture: a) 5%, in pure $CHCl_3$; b) 5%, in HFIP/$CHCl_3$ mixture (50:50, v/v); c) 10%, in pure $CHCl_3$; d) 10%, in HFIP/$CHCl_3$ mixture (50:50, v/v); e) 20%, in pure $CHCl_3$; f) 20%, in HFIP/$CHCl_3$ mixture (50:50, v/v); g) 20%, in HFIP/$CHCl_3$ mixture (75:25, v/v); h) 20%, in pure HFIP.

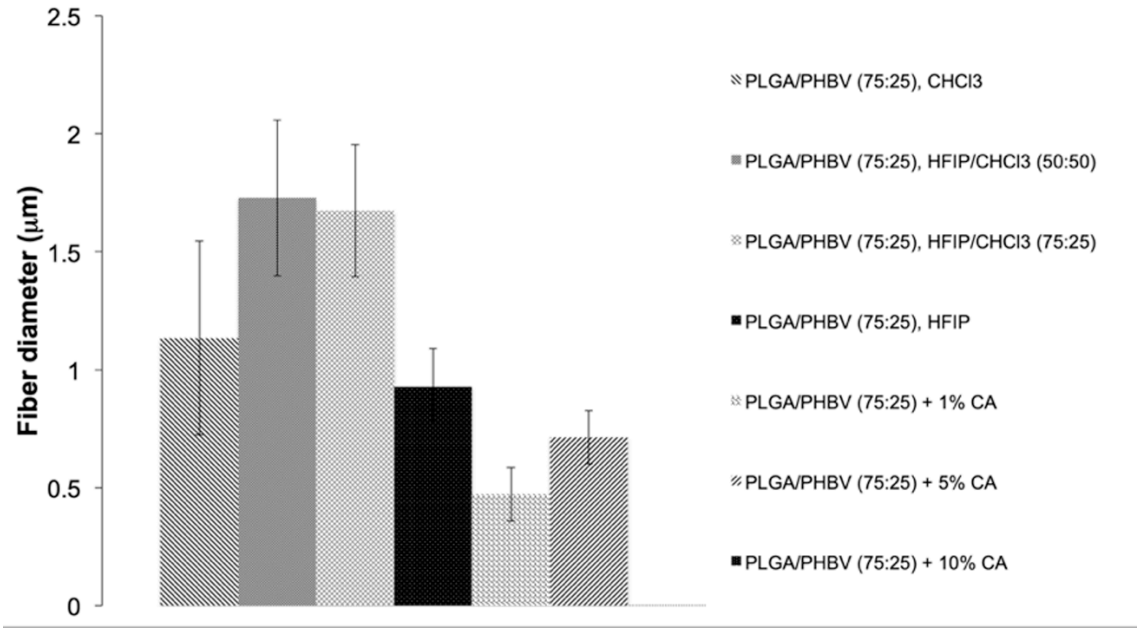

Figure 3.1.2 The average fiber diameter of electrospun PLGA/PHBV (75:25, w/w) membranes with and without CA. Error bars represent ± SD.

Among these electrospun membranes, we selected PLGA/PHBV (75:25, w/w) blend membrane dissolved in pure HFIP for CA loading experiments, since its fibers oriented more homogeneously with narrower diameter distribution. Besides, HFIP is a commonly used organic solvent, which is known from the literature that it can be entirely evaporated after the electrospinning process without leaving any residue on the fibers [75]. Different amounts of CA extract (1% and 5%, 10% w/v) were added to PLGA/PHBV (75:25, w/w) solution prepared from pure HFIP, and the polymer solutions with CA extract were electrospun under the conditions as mentioned earlier. Figure 3.1.3 shows representative SEM images of PLGA/PHBV electrospun membranes containing different amounts of CA extract. The average diameter of CA loaded PLGA/PHBV membranes were calculated 0.471±0.11 μm for PLGA/PHBV membrane with 1% CA amount and 0.714±0.11 μm for PLGA/PHBV membrane with 5% CA amount via Image J software (Figure 3.1.2). According to the SEM images, we observed that the fiber diameters were decreased after adding CA to the polymer solutions, which complies with the results of the previous studies [76]. As the ratio of CA increased to 5%, some deformations on fibers were observed, and the diameter distribution became wider comparing to the neat membrane. When we increased the CA ratio to 10%, the viscosity of the PLGA/PHBV blend solution became very low. Thus, the PLGA/PHBV blend solution containing 10% CA extract was not appropriate for electrospinning. Membranes electrospun from a polymer solution with an insufficient viscosity may become unstable and exhibit an inconsistent morphology [69]. According to SEM images of CA loaded PLGA/PHBV membranes, we eventually decided to use the electrospun PLGA/PHBV (75:25, w/w, 20% w/v) membrane having 1% of CA (w/v) with final concentration of 20% (w/v) extract as lower layer of the wound dressing material because of its appropriate fiber morphology. Hereafter, in this manuscript, the electrospun PLGA/PHBV (75:25, w/w, 20% w/v) membrane containing 1% of CA (w/v) with the final concentration of 20% (w/v) will be represented as electrospun PLGA/PHBV membrane with CA.

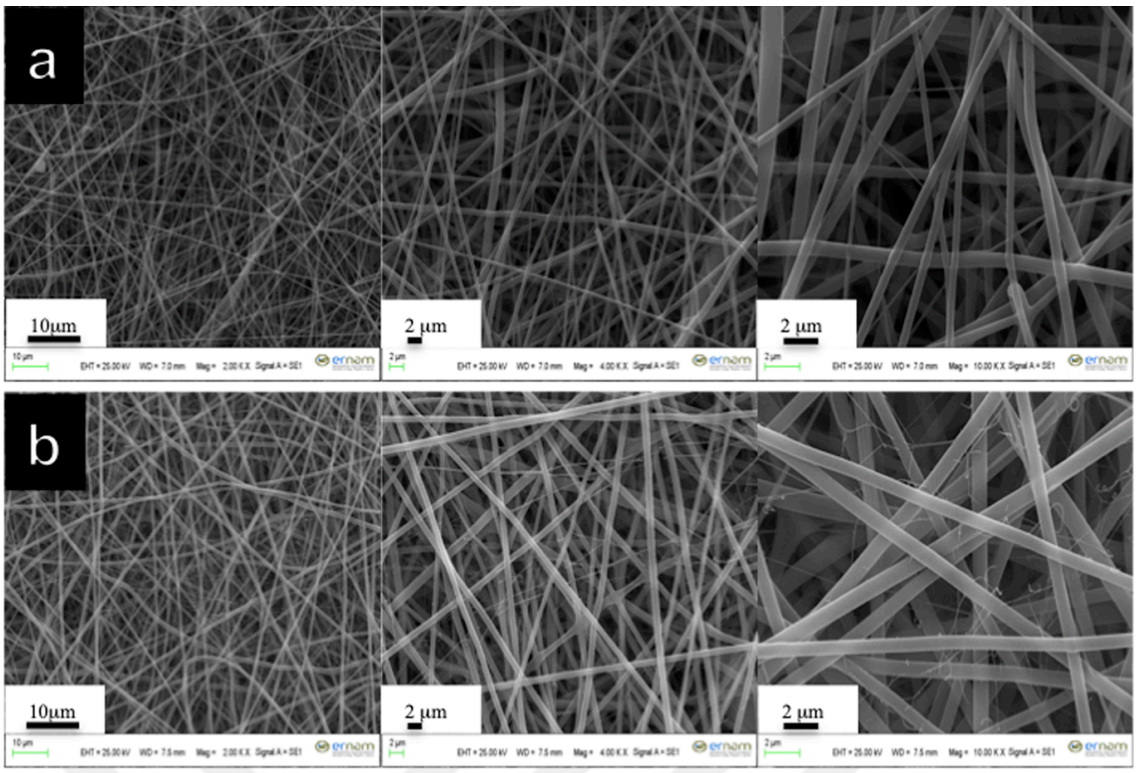

Figure 3.1.3 SEM images of electrospun PLGA/PHBV (75:25, w/w) membrane containing CA extract: a) 1% (w/v); b) 5% (w/v).

Upper Layer

The upper layer of the bilayered wound dressing was prepared from electrospun quaternized poly(4-vinyl pyridine) that we designed in our previous study [70] since it possesses nanofibrous structure and natural antibacterial characteristics because of quaternary ammonium groups through the polymer chain. Before electrospinning, we first synthesized poly(4-VP) and conducted 8 days-long quaternization process, which was confirmed via FT-IR spectrum and discussed detailed in the structural analysis part of the manuscript below.

After characterization, poly(Q-4VP) solutions were prepared at different concentrations (40, 50, 60 and 70 w/v) in DMF and electrospun with operating conditions of 18 kV and 1 ml/h of flow rate with a fixed tip-collector distance of 15 cm. Figure 3.1.4 represents SEM images of electrospun poly(4-VP) membranes at different concentrations. It can be seen that some bead formation was observed at 40% and 50% concentrations, while uniform and smooth nanofibers with very narrower diameter distribution were obtained at 60% and 70% concentrations. Image J software was also used for calculating the average diameters of fiber by measuring the diameters of randomly selected 50 fibers in the SEM images. The average fiber diameter of electrospun poly(Q-VP) membranes were calculated as 0.239±0.03 µm (at 40%

concentration), 0.263±0.04 µm (at 50% concentration), 0.460±0.057 µm (at 60% concentration) and 0.862±0.187 µm (at 70% concentration), respectively (Figure 3.1.5).

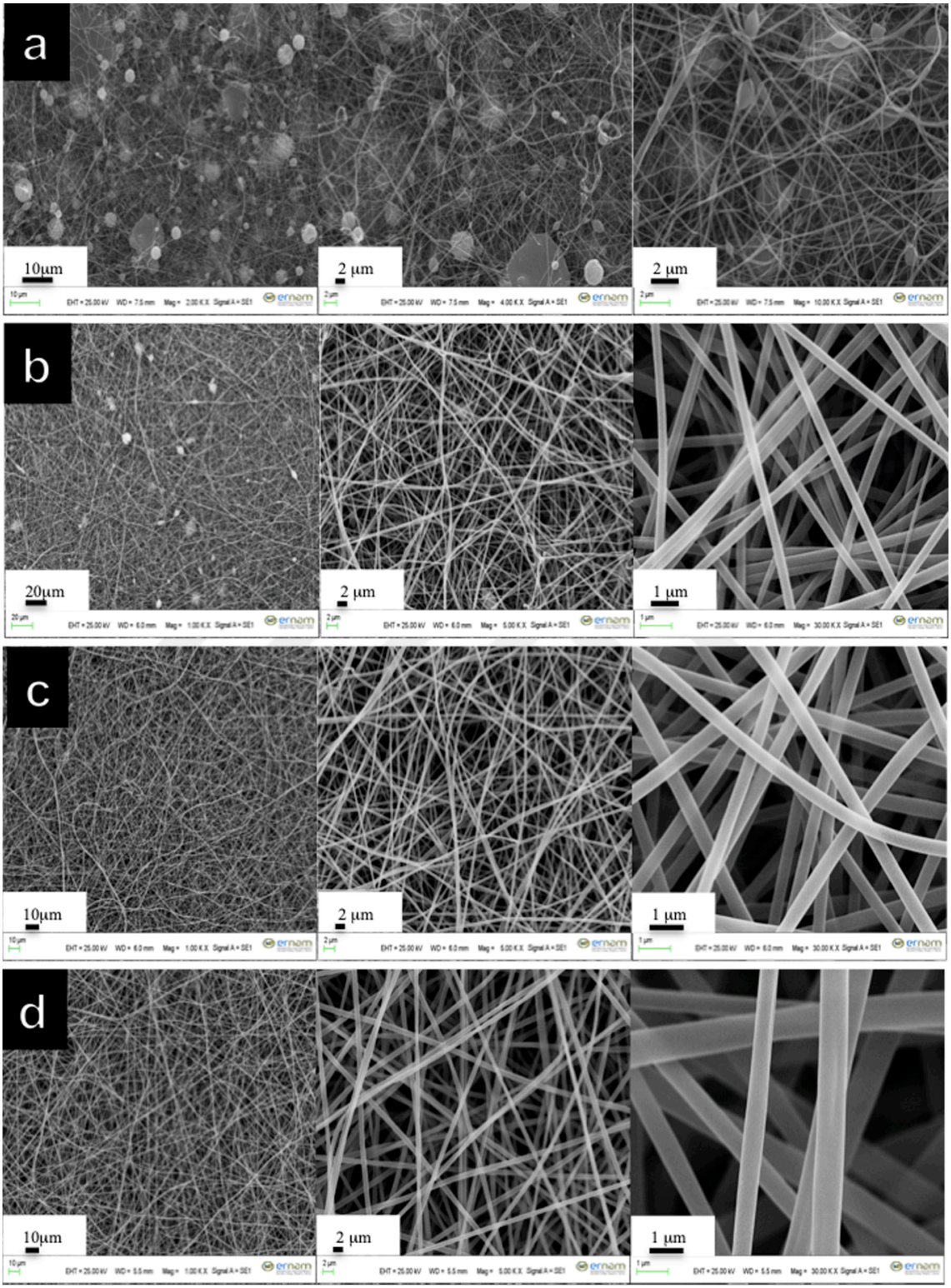

Figure 3.1.4 SEM images of poly(Q-4VP) at varying concentrations: a) 40%; b) 50%; c) 60%; d) 70%.

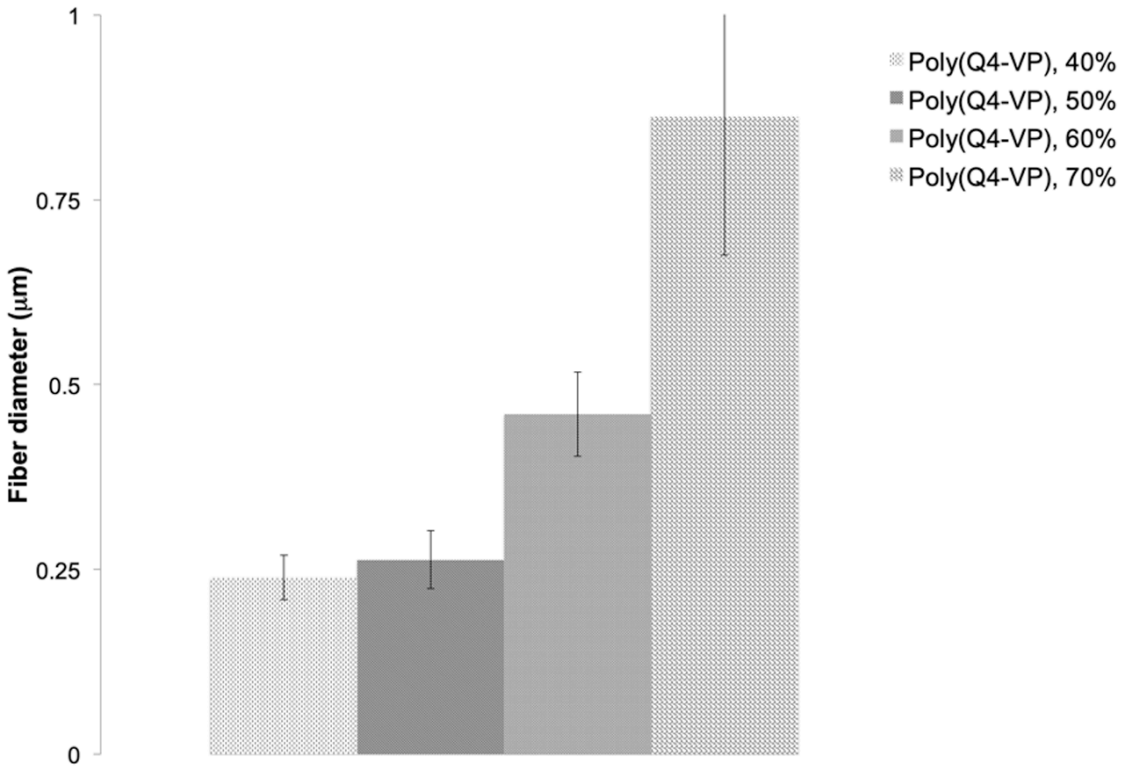

Figure 3.1.5 The average fiber diameter of electrospun poly(Q4-VP) membranes at different concentrations. Error bars represent ± SD.

Among resulting membranes, we selected electrospun poly(Q4-VP) with the concentration of 60% as the upper layer of bilayered electrospun mat, since it possesses uniform nanofiber morphology with narrower diameter distribution. Hereafter, in this manuscript, the electrospun eight days long quaternized poly(4-VP) with a final concentration of 60% (w/v) will be represented as poly(Q-4V). Eventually, we obtained CA containing bilayered electrospun wound dressing (Figure 3.1.6).

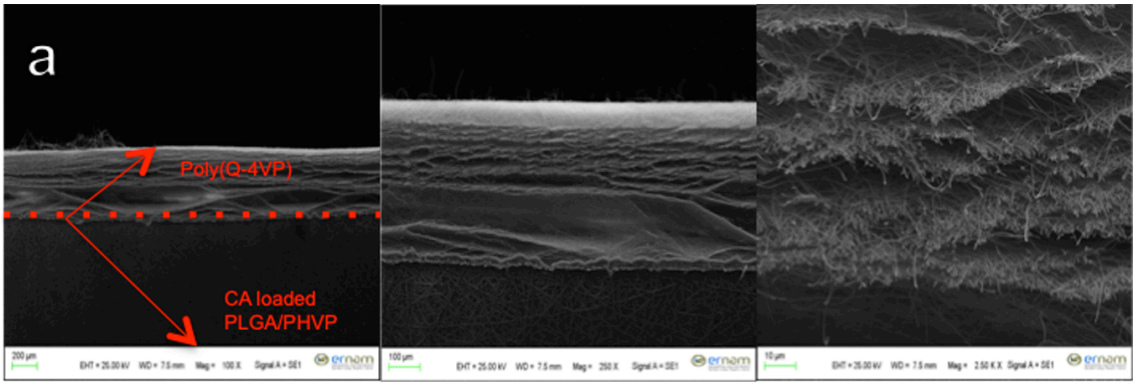

Figure 3.1.6 SEM images of CA containing bilayered electrospun membrane with electrospun poly(Q4-VP) upper layer and CA loaded electrospun PLGA/PHBV lower layer.

3.2 FT-IR Analysis

Lower Layer

FT-IR spectra of pure PLGA, PHBV, electrospun PLGA/PHBV membrane, CA loaded electrospun PLGA/PHBV membrane and, neat CA extract are shown in Figure 3.2.1. From the spectrum of PLGA samples, it can be seen that an active C=O stretching band at 1750 cm^{-1} existed [78]. PLGA showed several characteristic peaks at 2990 and 2940 cm^{-1}, 1183, and 1083 cm^{-1} that were assigned to $–CH_3$, and C–O stretching, respectively [79]. In the spectrum of PHBV, the absorption of the saturated ester C=O groups appeared at 1720 cm^{-1}. The absorption peak at 1276 cm^{-1} in the FT-IR spectrum of PHBV indicates the strong C-O stretching band, which further confirms the presence of the ester functional group [77]. All the characteristic peaks belong to PLGA and PHBV appeared both in the spectra of electrospun PLGA/PHBV and CA loaded electrospun PLGA/PHBV membranes. These FT-IR spectra confirmed that both PLGA and PHBV integrated with the structure and electrospinning did not cause any change in the chemical structure of the blend membrane.

In the FT-IR spectrum of neat CA, the peak at 3271 cm^{-1} indicates the presence of carboxylic acid with O–H stretching vibrations, while the peaks at 2926, 1645, 1452 and 1043 cm^{-1} indicate the presence of alkyl groups, alkenes, alkyl halides, and chloroalkanes in the extract of CA, which were shown to be similar to the previous studies [80, 81]. As a meager amount of asiaticoside was added into the electrospun membrane, the peaks belong to CA extract did not appear in the spectrum of CA extract containing PLGA/PHBV electrospun membrane. The FT-IR results coincide well with the previous studies in the literature.

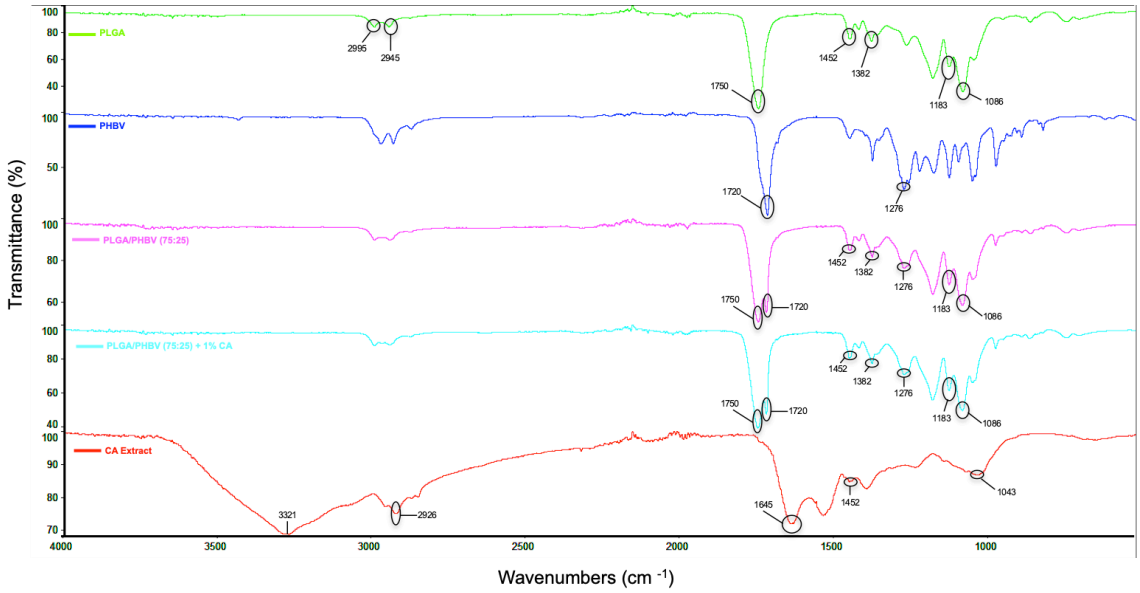

Figure 3.2.1 FT-IR spectra of PLGA, PHBV, electrospun PLGA/PHBV membrane, electrospun PLGA/PHBV membrane with CA and, neat CA extract.

Upper Layer

The upper layer of the bilayered wound dressing was prepared from electrospun quaternized poly(4-vinyl pyridine). To do so, we first synthesized poly(4-VP) by random radical polymerization of 4-VP and characterized by the FT-IR analysis (Figure 3.2.2). According to the spectrum, the characteristic peaks of poly(4-VP) at 3057, 1670, 1450, and 1165 cm^{-1} may be assigned to aromatic C-H stretching, aromatic C=C stretching, and C-N stretching [82]. The comparison of the spectrum of poly(4-VP) to the spectrum of poly(Q4-VP), the new peaks appeared at 1640 and 1720 cm^{-1}, which may be assigned to quaternary ammonium and carboxylic acid groups (from caproic acid), respectively [83]. The peak at 3400 cm^{-1} also supported that caproic acid was associated with polymer structure resulting quaternization of the pyridine unit. We calculated the quaternization degree as 45% by using absorbance values of the peaks for poly(4-VP) and quaternary form (1670 and 1720 cm^{-1}) according to the method given in literature 70). Thus, the FT-IR spectra confirm the quaternization of poly(4-VP), which is a kind of approval of antimicrobial property as we have already tested by our group before. In our previous study, we applied eight days of the quaternization process and achieved antimicrobial characteristics. In this study, as we used the same quaternary polymer, we applied the same procedure to obtain quaternized polymer with determined antimicrobial characteristics [70].

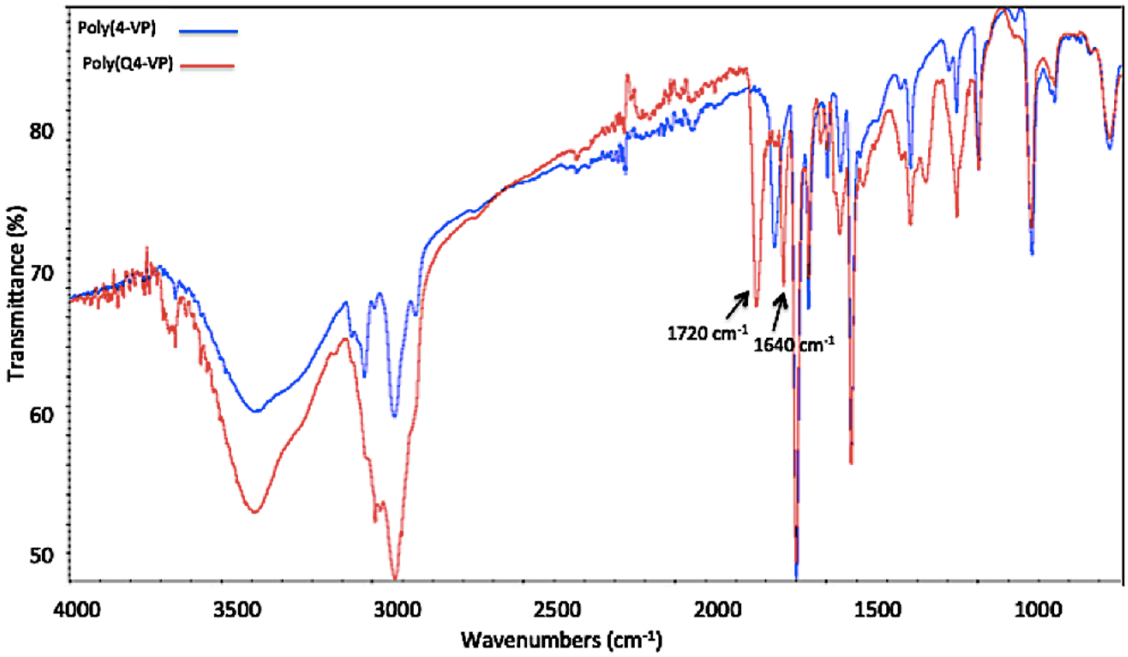

Figure 3.2.2 FT-IR spectra of neat poly(4-VP) and quaternized poly(4-VP).

3.3 Thermal Analysis

The thermal properties of pure PLGA, PHBV, and electrospun PLGA/PHBV membrane were investigated by using DSC (Figure 3.3.1). According to the DSC thermograms, the amorphous nature of PLGA as it only shows the glass transition temperature (Tg) 47.86 ^{o}C. The standard double melting temperatures of pure PHBV were observed at 144.17 and 155.39 ^{o}C due to the formation of various crystals and their melting, recrystallization, and remelting, which was previously reported in the literature [84, 85]. For electrospun PLGA/PHBV blend membrane, double melting peaks were also appeared at 145.66 and 155.43 ^{o}C, while single peak attributed to Tg of PLGA was seen at 46.09 ^{o}C, which is a bit lower than that Tg of PLGA due to improvement in the orientation of molecular chains in the electrospun and as well as the larger area to volume ratio of electrospun fibers [86]. These results indicated the miscibility of pure PLGA and PHBV in the electrospun PLGA/PHBV blend membranes that can be determined by observation of the glass transition temperature of the blend [85].

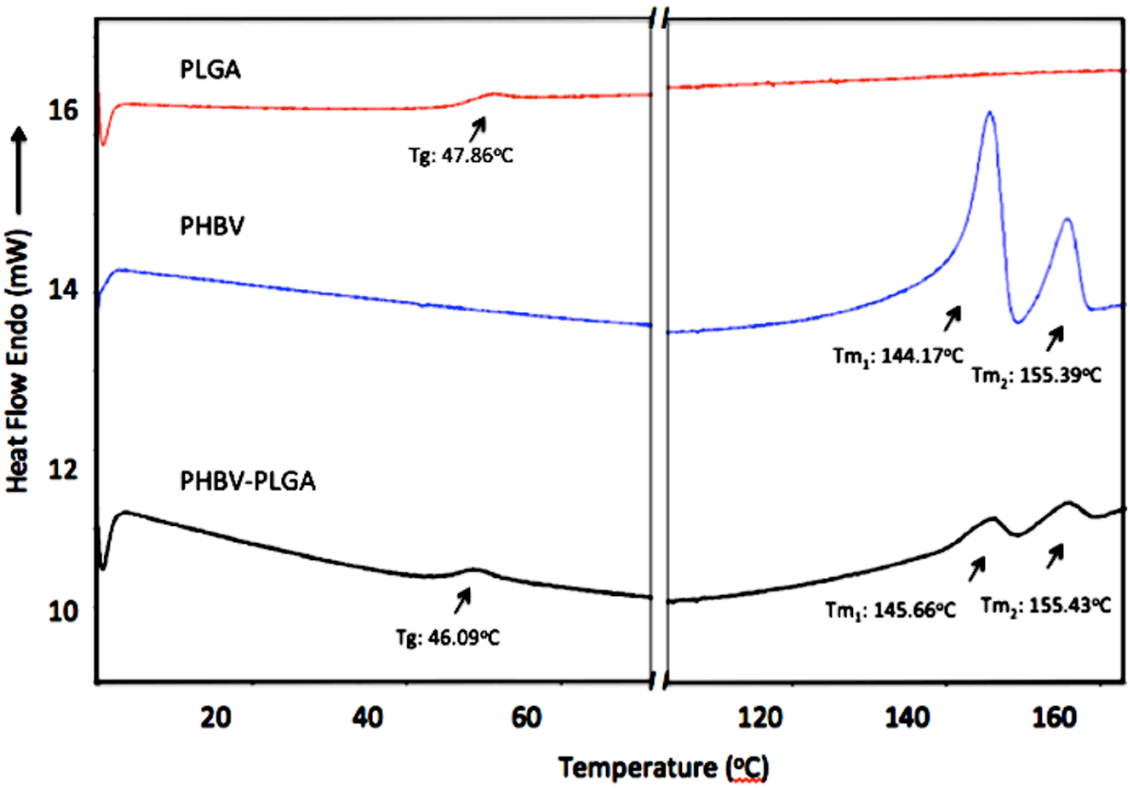

Figure 3.3.1 DSC thermograms of electrospun PLGA, PHBV, and PLGA/PHBV blend membrane.

3.4 Absorption of Wound Fluid

Absorption of excessive wound exudate is one of the most critical characteristics of an ideal wound dressing material. Thus, the absorption capacity of the lower layer of the bilayered wound dressing, which contacted the wound directly, was investigated and PLGA/PHBV membrane without CA and PLGA/PHBV film prepared by solvent casting method were used as controls. PECF was used as wound fluid, and the absorption values for the membranes were obtained as 437% and 420% for electrospun membranes with and without CA, respectively (Figure 3.4.1). Non-fibrous of PLGA/PHBV film revealed only 4% absorption, and this value was consistency with the literature [87]. According to the results, CA containing membrane had a better absorption capacity than electrospun PLGA/PHBV membrane indicating the better exudate absorption capacity.

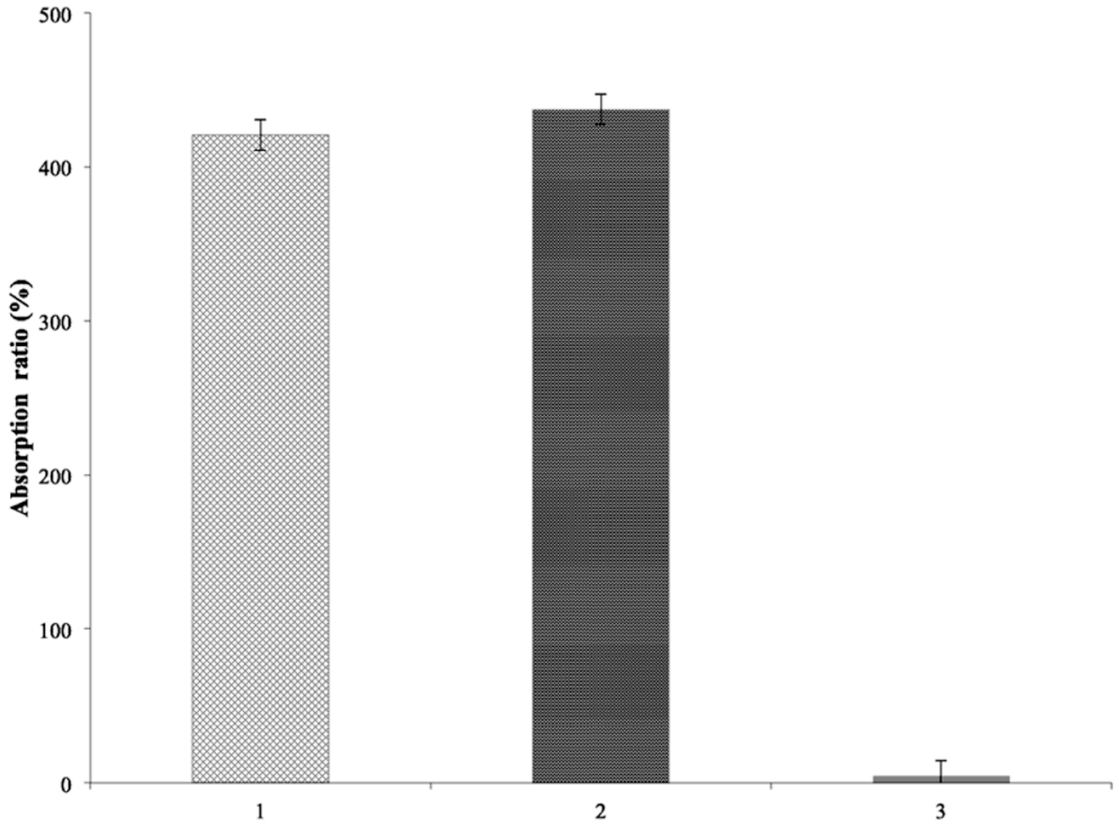

Figure 3.4.1 The absorption capacity of membranes: 1) Electrospun PLGA/PHBV membrane, 2) Electrospun PLGA/PHBV membrane with CA, 3) Non-fibrous film of PLGA/PHBV blend. Data points are the average of n=3, and error bars represent ± SD.

3.5 Mechanical Analysis

Typical stress-strain curves of neat PLGA, PHBV, and PLGA/PHBV electrospun membranes with and without CA at an initial strain rate of 1 x 10^{-1} s^{-1} are given in Figure 3.5.1. Our results indicate that the neat PLGA membrane showed the best strength value with plastic deformation and necking, while neat electrospun PHBV membrane showed the lowest strength and ductility values. Ultimate tensile strength values were found 1.86 MPa for PLGA/PHBV membrane without CA and 1.62 MPa for PLGA/PHBV membrane with CA, respectively. It can be seen that the electrospun PLGA/PHBV blend membrane with CA showed slightly lower strength values and ductility compared to the CA-free membrane. In our previous study working with the scientists from the mechanical engineering field, we investigated the specific mechanical behavior of the PLGA/PHBV and PHBV/PCL electrospun nonwoven mats with and without CA and demonstrated the effects of CA on the electrospun membranes' mechanical behavior at different strain rates [88]. According to the results,

we observed that adding CA to the PLGA/PHBV electrospun membrane decreased the strength value with enhancing ductility slightly compared to the neat PLGA/PHBV at the different strain rates, as we also demonstrated in our recent study. The value for the ultimate tensile stress that we found in our recent study is 1.62 MPa, and it corresponds to 7.864 MPa as Young's Modulus. According to literature, the ultimate strength and Young's Modulus value indicated in our study is very similar to the values of the native skin tissue, whose Young's Modulus value is 4.6-20 MPa [89]. Thus, we can conclude that the mechanical property of the CA extract containing bilayered electrospun wound dressing designed in our study is adequate to be used for wound healing applications.

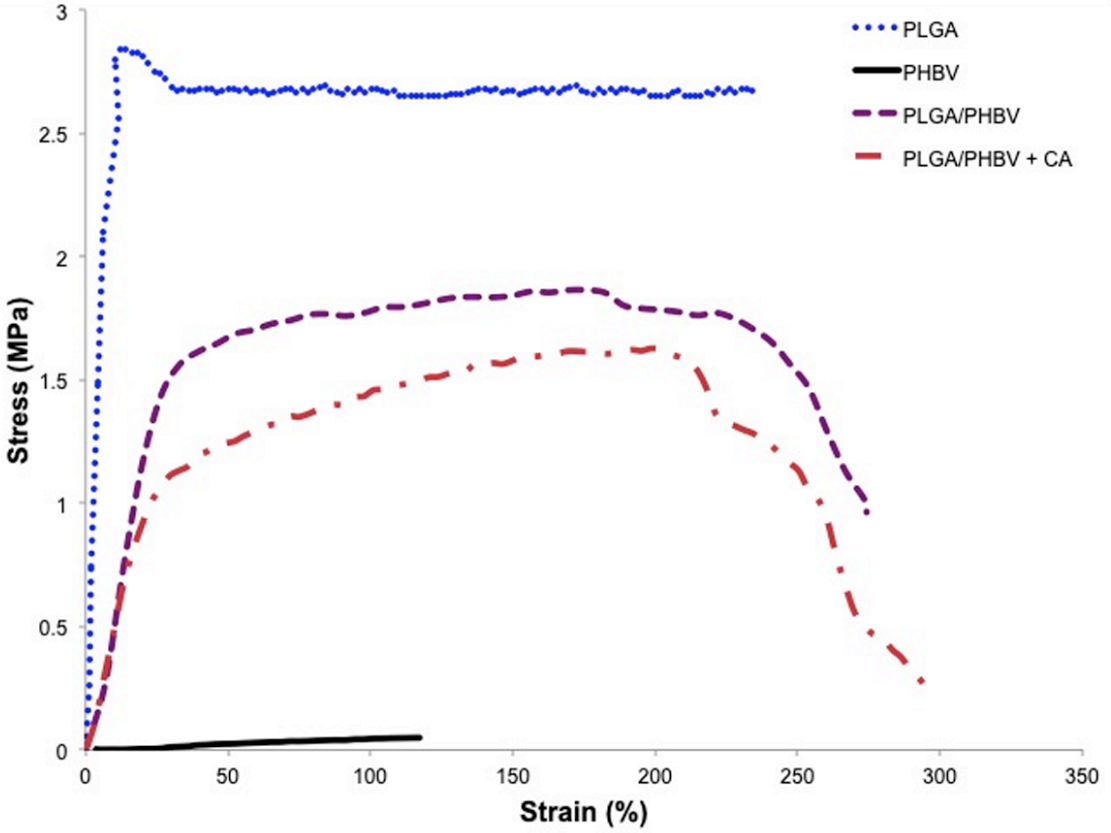

Figure 3.5.1 Typical stress-strain curves of electrospun PLGA, PHBV, and PLGA/PHBV membranes with and without CA.

3.6 *In vitro* release profile of CA

In the literature, Gupta et al. showed that asiaticoside, an essential compound in CA, has a specific absorbance peak at 215 nm [90]. Therefore, we monitored the amount of asiaticoside using the absorption peak at 215 nm for calculating the *in vitro* cumulative release of CA extract from electrospun PLGA/PHBV membrane. According

to our result presented in Figure 3.6.1, the extract was rapidly released *in vitro* from the electrospun membrane in the first 5 hours, followed by a slow release over a prolonged period (24 h). Zhu et al. investigated the *in vitro* drug release profile of asiaticoside loaded electrospun alginate/PVA/chitosan membrane and showed that asiacoside released from the membrane in 24 h [91]. Also, Suwantong et al. performed release study of asiaticoside from herb-loaded CA fiber mats, and they used two different types of release medium, which are 10% methanol consisting of acetate buffer and PBS buffer. According to their result, asiaticoside release from the membrane in acetate medium and PBS medium reached plateau value at 720 and 480 minutes after immersion, respectively [92]. The *in vitro* release profile of CA from electrospun PLGA/PHBV membrane given herein is consistent well with previous studies. Based on the results, we conclude that the rapid release of CA from the membrane may be useful for the wound healing process.

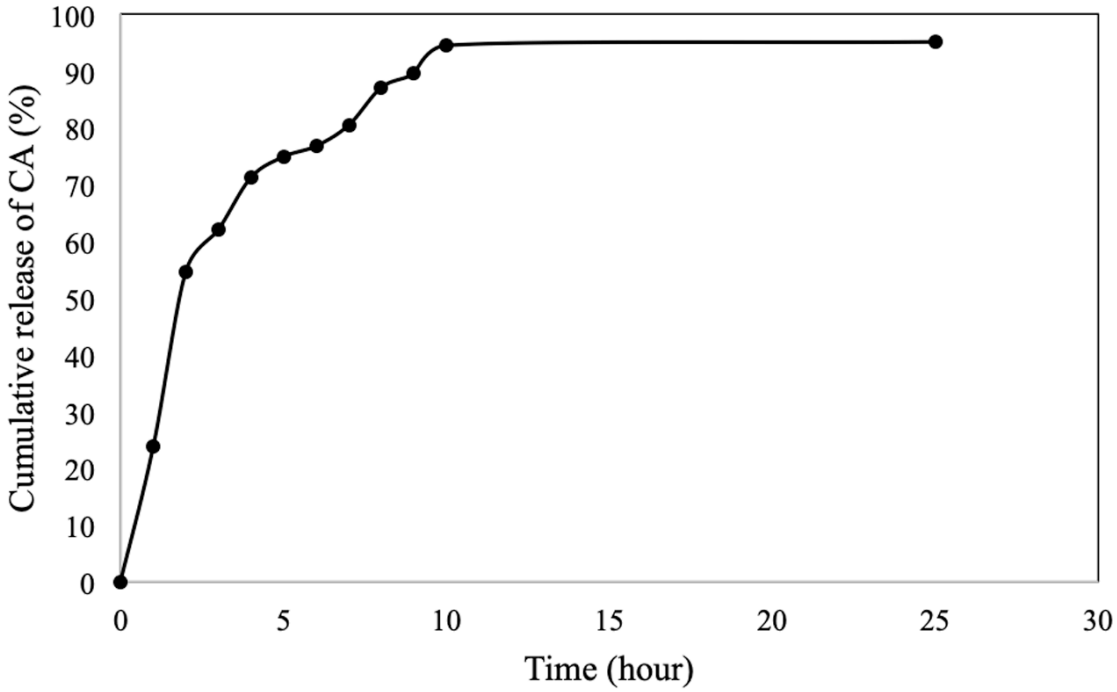

Figure 3.6.1 *In vitro* cumulative release profile of CA from electrospun PLGA/PHBV membrane.

3.7 Assessment of Cell Viability

MTS assay was conducted to evaluate the biocompatibility of electrospun membranes. Figure 3.7.1 presents the results. Membranes showed no significant differences in cytotoxicity at days of culture. Cell viability was calculated >95% for all membranes, which approves that these membranes have not cytotoxic effect. Moreover,

1% of CA content did not cause any toxicity to the membranes as well as poly(Q-4VP) in the applied dose. PLGA, PHBV, and poly(Q-4VP) are biocompatible and biodegradable polymers that have been widely investigated as wound dressing materials, as mentioned before.

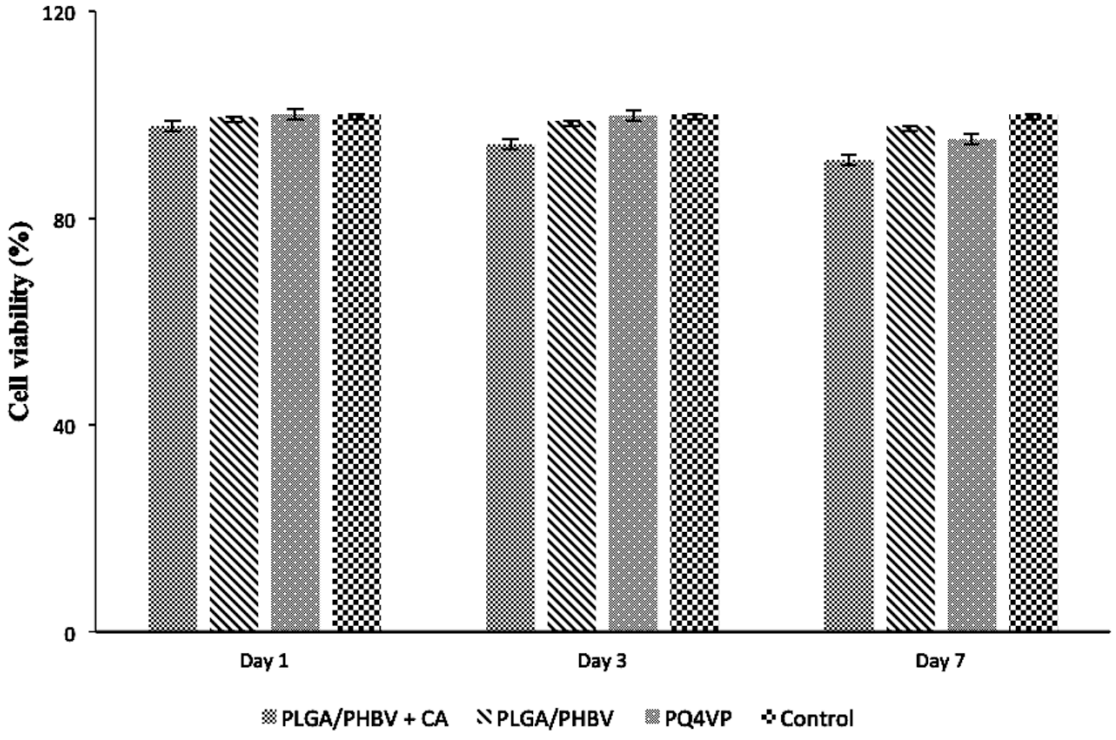

Figure 3.7.1 Viability (%) of human fibroblast cells on electrospun membranes following (A) 1 day or (B) 3 days and (C) 7 days of culture. Data points are the average of n=3, and error bars represent ± SD.

3.8 Assessment of Cell Proliferation and Attachment

We tested the proliferation and attachment of human fibroblast cells seeded on electrospun PLGA/PHBV membranes with and without CA by utilizing SEM on days 1, 4, and 7. According to SEM images, fibroblasts adhered to both membranes and stretched across the fibrous substrates upon proliferation. As shown in Figure 3.8.1, the density of viable cells on both membranes was observed almost identical. Moreover, after seven days of cell culture, cell attachment on electrospun PLGA/PHBV membrane containing CA was better than that on neat electrospun PLGA/PHBV membrane. Previous studies reported that CA supports cell migration, proliferation, and growth [6, 34-35]. Moreover, the appropriate surface morphology of fibers is one of the essential

requirements for biocompatibility [2]. Thus, based on the SEM images, the surface and morphology of electrospun membranes are appropriate for skin reconstruction.

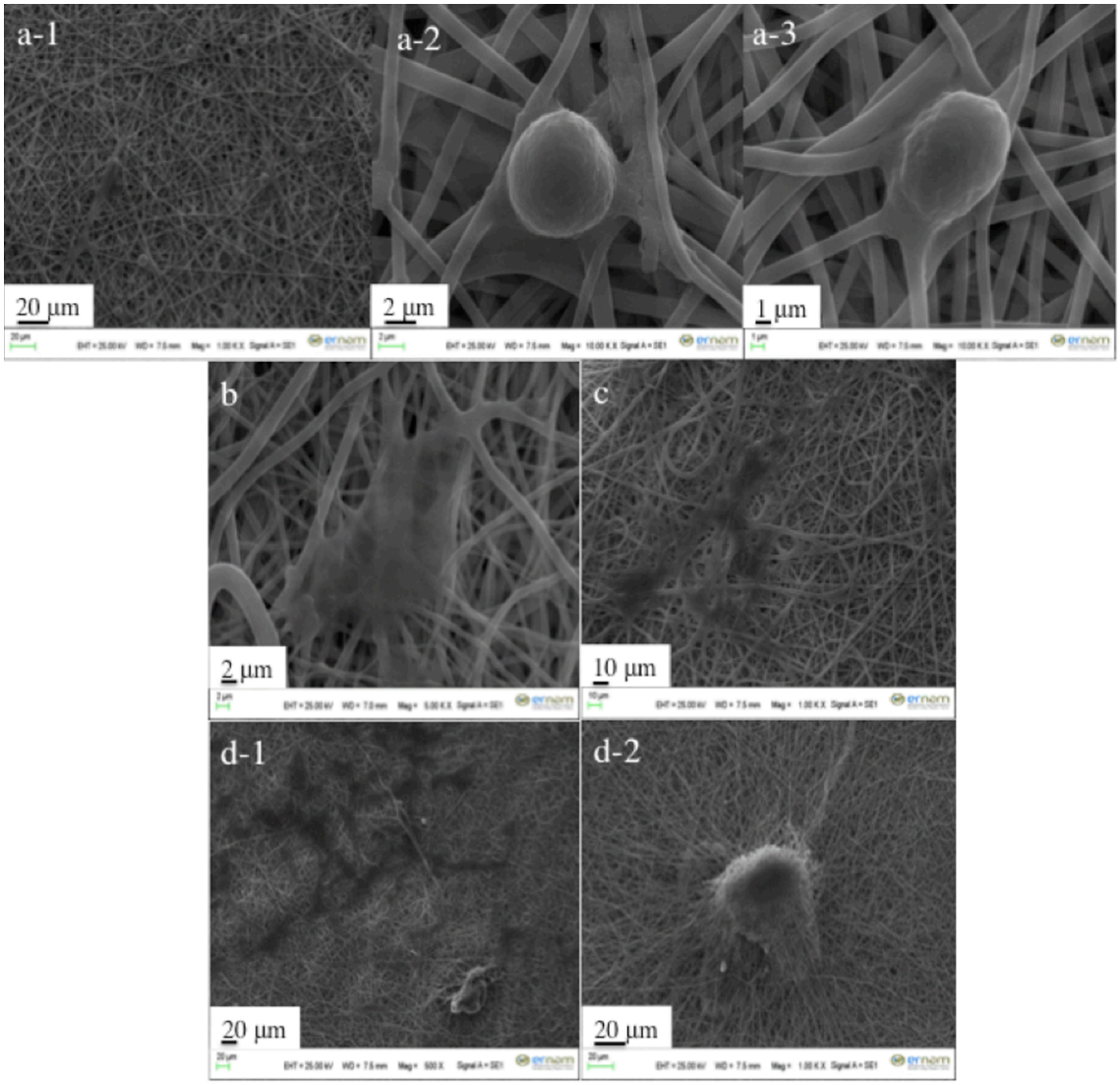

Figure 3.8.1 SEM images of the proliferation and attachment of human fibroblast cells: a) on PLGA/PHBV electrospun membrane without CA, day 1; b) day 4; c) day 7; d) with CA, day 7.

Chapter 4

CONCLUSIONS AND FUTURE WORK

In our study, we successfully fabricated a novel antimicrobial bilayered wound dressing with a three-dimensional porous structure similar to that of the native ECM via electrospinning. The lower layer of the wound dressing, which directly contacted the wound, was designed from CA (1%, w/v) containing electrospun PLGA/PHBV blend (75:25, w/w), while the upper layer was prepared from poly(Q-4VP) (60%, w/v) by sequential and continuous electrospinning on the lower layer. Poly(4VP) was chemically modified to obtain quaternary form based on our previous study, in which antimicrobial characteristic of the poly(Q-4VP) was proved against the E. coli and S. aureus. Our results also demonstrated the significant effect of the lower layer of the wound dressing on the adhesion and proliferation of human fibroblast cells. Based on our results, the CA containing electrospun bilayered wound dressing fabricated and characterized in this study could be an alternative to conventional wound care applications.

Every year, over 6 million people are estimated to be suffered from different types of wounds and skin injuries as a result of diseases, surgical operations, and accidents, and the number of victims and patients is increasing exponentially in industrialized countries. Thus, the need and demands on bioactive wound dressings are increased due to the lack of conventional wound dressings in terms of accelerating wound healing processes and preventing bacterial attacks. Based on these facts, we will conduct animal studies of the electrospun bilayered wound dressing designed and characterized in this study to show the in vivo performance on the wound healing process. Besides, we are planning to investigate the electrospun nanofibrous membrane prepared from different blend polymers incorporating different types of active agents.

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