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M1 to M2 Macrophage Induction Using Retinoic Acid and Mesenchymal Stem Cells Loaded on an Electrospun Pullulan/ Gelatin Scaffold To Promote Healing of Chronic Wounds

Kaivon Assani
Wright State University

M1 TO M2 MACROPHAGE INDUCTION USING RETINOIC ACID AND MESENCHYMAL STEM CELLS LOADED ON AN ELECTROSPUN PULLULAN/GELATIN SCAFFOLD TO PROMOTE HEALING OF CHRONIC WOUNDS

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By

KAIVON ASSANI

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Wright State University

WRIGHT STATE UNIVERSITY

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Kaivon Assani ENTITLED M1 to M2 Macrophage Induction Using Retinoic Acid and Mesenchymal Stem Cells Loaded on An Electrospun Pullulan/Gelatin Scaffold to Promote Healing of Chronic Wounds BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science in Biomedical Engineering.

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Jaime Ramirez-Vick, Ph.D.
Thesis Director

Committee on Final Examination

_____

Jaime Ramirez-Vick, Ph.D.
Department Chair

_____

Jaime Ramirez-Vick, Ph.D.

_____

Nasim Nosoudi, Ph.D.

_____

Amir Zadeh, Ph.D.

_____

Barry Milligan, Ph.D.
Interim Dean, Graduate School

ABSTRACT

ASSANI, KAIVON. M.S.B.M.E. Department of Biomedical, Industrial and Human Factors Engineering, Wright State University, 2018. M1 to M2 Macrophage Induction Using Retinoic Acid and Mesenchymal Stem Cells Loaded on An Electrospun Pullulan/Gelatin Scaffold to Promote Healing of Chronic Wounds

Modulation of macrophage polarization is required for effective tissue repair and regenerative therapies. Conversion of macrophages from inflammatory M1 to fibrotic M2 phenotype could help in diseases such as chronic wound which are stuck in inflammatory state. During the inflammatory phase, macrophages are of the inflammatory phenotype (M1) and distribute pro-inflammatory cytokines including TNF-α and IL1β which are microbicidal and recruit/activate cells. In normal wound healing macrophages then switch to a fibrotic phenotype (M2) promoting wound closure by angiogenesis, and matrix deposition. Chronic wounds are a major biological and financial burden to both patients and the health care system, costing over $25 billion to Medicare annually. Natural wound healing proceeds through several largely overlapping phases that involve an inflammatory response and associated cellular migration, proliferation, matrix deposition, and tissue remodeling. The initial stages of the inflammatory response are dominated by neutrophils followed soon after by macrophages, which become prominent at the wound site. A sustained inflammation is an important aspect in the disruption of the normal healing process that can lead to a chronic condition. The chronic conditions start when the highly

phagocytic M1 macrophages are done removing any infected or non-functional cells, and any damaged matrix or foreign debris and do not differentiate into an M2 phenotype. Thus, inducing these sustained M1 macrophages to differentiate into an M2 phenotype should correct this condition, and has been shown to improve wound healing.

We suggest simultaneously using retinoic acid (RA) and mesenchymal stem cells (MSCs) to promote M1 to M2 transition. RA and MSCs have both shown to promote M1 to M2 transition, and in addition, MSCs can promote wound regeneration. **We hypothesize that treating M1 macrophages with retinoic acid and mesenchymal stem cells loaded on a pullulan/gelatin scaffold will promote M1 to M2 conversion**. To facilitate this, we developed an electrospun hydrogel consisting of 75% pullulan and 25% gelatin and crosslinked with 1:70 ethylene glycol diglycidyl ether (EGDE) in ethanol (EtOH). Pullulan was chosen due to its ability to quench reactive oxygen species and reduce inflammation, as well as for its excellent mechanical properties. While gelatin was added to provide functional motifs for cellular attachment. The scaffold composition was determined via FTIR. The scaffold degraded to approximately 80% after 14 days, and approximately 38% of the drug was released after 7 days. Scaffold nanofibers were determined to 328nm (±47.9) in diameter. RA and MSCs were directly loaded and used to treat M1 THP1 cell derived macrophages to induce polarization. qPCR shows a reduction of M1 markers TNFα and IL1β, and an increase of M2 marker CCL22 after 2 days of scaffold treatment, suggesting successful M1 to M2 transition.

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Dedication

This thesis is dedicated to my mother, Teresa, who was caring and always there for me, to my father, Behzad, who provided for us and showed me how to be a good person, to my brothers Keavash and Korash who taught me humility and how to teach, and to my loving and supporting wife, Lisa, for whom I would not be here without.

CHAPTER I

Introduction

Macrophages are critical immune cells involved in controlling infection, inflammation and disease. These cells are multifunctional and can be highly plastic, able to switch between phenotypic expression patterns depending on environmental ques [1]. Macrophages play a role in both the adaptive and innate immune system. Their role in the adaptive immune system involves being antigen presenting cells. In addition, their induction of regulatory T cells plays a role for tissue regeneration and disease prevention [2]. Here we will mainly focus on their role within the innate immune system, as it relates to their response to bacterial infection and tissue injury.

During wound healing macrophages migrate to the site of injury from the blood as well as from the surrounding tissues. Macrophages initially become inflammatory to promote the clearance of infection and debris. They secrete matrix metalloproteinases (MMPs) to remove damaged cells and make way for scar formation. Normally macrophages contribute to disease prevention, infection removal, debris removal, tissue

healing and regulation, however if regulation of wound healing does not occur appropriately a chronic inflammatory condition can develop [3]. This would promote a consistent M1 phenotype for the macrophages.

Macrophages are commonly described as one of two phenotypes inflammatory M1 or fibrotic/anti-inflammatory M2 [4]. Regulation between M1 and M2 phenotypes is critical to manage infection and disease [5]. M1 macrophages produce inflammatory cytokines which recruit immune cells, such a neutrophils, promoting further inflammation. During normal healing processes M1 macrophages are predominant to removing debris, infection and damaged cells. M2 macrophages down regulate inflammation and promote tissue deposition. Dysregulation of M1 or M2 expression has been associated with inflammatory diseases including chronic infection, chronic wounds (i.e., diabetic, pressure and venous ulcers), asthma, cancer, Parkinson's, chronic obstructive pulmonary disease, atherosclerosis, Alzheimer's disease and cancer [6]–[12]. This study aims to promote conversion of M1 macrophages to M2 macrophages which could serve as a treatment for chronic inflammatory diseases, which show high M1 macrophage populations.

In our project we developed an electrospun hydrogel composed of pullulan and gelatin, loaded with retinoic acid (RA) and adipose derived mesenchymal stem cells (ADMSCs) to promote M1 to M2 conversion in macrophages. Both RA and ADMSCs have been shown to polarize macrophages to M2 phenotype as well as fibrosis. This patch was designed with chronic wounds in mind taking into account studies which have used pullulan, gelatin, RA and MSCs in separate experiments to improve chronic wound healing

[13]–[16]. This is the first time each of these has been used together. Such a therapeutic could improve healing of chronic wounds which affect over 8.2 million patients on Medicare and cost over 25 billion dollars per year [17], [18].

When macrophages arrive at the site of inflammation they are exposed to inflammatory stimulants such as lipopolysaccharide (LPS) and interferon gamma (IFN-γ) which causes polarization towards the M1 phenotype. These macrophages then further stimulate inflammation by releasing inflammatory cytokines including TNF-α and IL1β, as well as other microbicidal molecules such as MMPs. At the end of the inflammatory phase, M1 macrophages differentiate into M2 macrophages, which express anti-inflammatory cytokines, including CCL18 and CCL22.

Macrophage role in adaptive immunity also depends on their polarization. M1 macrophages are antigen presenting cells which recruit and communicate with T helper Th1 cells, while M2 macrophages coordinate Th2 cell recruitment and suppress Th1 cell response. Macrophages play a vital role in controlling infection, disease, regeneration and wound healing, and by promoting M1 to M2 transition inflammatory diseases be ameliorated [19]. [20], [21]

We hypothesize a novel combination of MSCs and RA loaded with electrospun nanofiber scaffold made of pullulan and gelatin as a therapeutic to promote M1 to M2 induction. Research has shown that ADMSCs and RA improve M1-M2 transition and wound repair, however, their joint effect has not been documented [13], [14], [22], [23]. Pullulan was chosen because it offers good biocompatibility and anti-inflammatory

properties by quenching reactive oxygen species (ROS) [15], [24]–[26]. Gelatin has good biocompatibility and binding motifs for cellular adhesion, making it an ideal copolymer. Gelatin is extensively used in biomedical engineering and has been approved for use in drug delivery and wound healing [27]. After electrospinning, ethylene glycol Diglycidyl ether (EGDE) was used to crosslink the nanofibers. It can bind to the hydroxyl group on both pullulan and gelatin to improve mechanical strength and delay degradation. Pullulan and gelatin has been shown to be effective scaffolds for wound healing [28]–[30]. This is the first study which combines MSCs, RA, pullulan and gelatin. Here we verify the structure and composition of the scaffold using FTIR and SEM, examine degradation and release rate, and evaluate the potential to modulate macrophage polarity from M1 to M2 phenotype using qPCR.

CHAPTER II

Background

2.1 Macrophages

Macrophages are immune cells derived from the myeloid lineage. They are located throughout the body and stationed in specific tissues to help recycle dead cells and clean away foreign debris and material. When there is no tissue damage, macrophages help to recycle apoptotic cells and around 200 billion dead erythrocytes per day [31]. This process is known as phagocytosis, and it is locally controlled in response to specific ques [32]. Macrophages also regulate the inflammatory response which is part of the response to cell death and debris. Imbalances in the inflammatory process result in cell and tissue damage such as in chronic inflammatory disease [33]-[34].

2.1.1 Macrophage Development and Specialization

Macrophages are replenished by bone marrow derived monocytes; however, tissue-specific macrophages may rely on a self-renewal process. Tissue specific

macrophages that self-renew originate from embryonic macrophages. Bone marrow-derived macrophages, on the other hand, provide macrophages on demand from monocyte precursors. Bone marrow-derived macrophages come from hematopoietic stem cells, which give rise to Ly6C^{hi} monocytes. These require CCR2 to be able to exit the bone marrow and enter the blood stream. Fate mapping activates important reporter genes to determine downstream lineage. Bone marrow monocytes can differentiate into many types including tumor-associated macrophages, monocyte-derived dendritic cells (e.g., during colitis, lung infection, etc.), effector monocytes (e.g., for colitis, peritonitis, liver disease), monocyte-derived macrophage (e.g., for tissue injury, such as skin, muscle, heart and central nervous system), and some tissue-resident macrophages (e.g., found in intestine, lung, mammary gland, skin, heart, osteoclasts). The tissue-resident macrophages derived from monocytes have a limited half-life and no self-renewal [3], [35].

2.1.2 Macrophages in Tissue

The mononuclear phagocyte cellular system can be considered the sum total of responses due to mononuclear cells, such as macrophages. It is adaptable and contributes to both, adaptive and innate immunity. All macrophages, including tissue specific and bone marrow-derived, are a part of it. Tissue-specific macrophages can be identified via morphology, histological staining, or labeling of phagocytic particles. A common marker for macrophage differentiation if the F4/80 antigen which is associated with endothelial and epithelial cells as well as distribution in organ interstitium and connective tissue [36], [37].

Macrophages have specific functions in the tissues they are localized in. For instance, stromal macrophages have been found to support erythropoiesis, spleen macrophages support cell turnover and innate and adaptive immunity, lung macrophages protect airways, peritoneal macrophages guard the abdominal serous cavity, and neural macrophages support development. Macrophages affect growth and development of many tissues. [38]

2.1.3 Macrophage Polarization

Macrophage polarization refers to the activation of certain sets of macrophage genes and deactivation of others. Macrophages are very plastic cells with the ability to have varying gene expression. They variation in expression is in response to many signals such as debris, tissue trauma and infection. Activated macrophages are generally categorized as M1 or M2 macrophages. M1 macrophages are inflammatory in phenotype and has toll like receptors and interferon signaling. These are associated with inflammatory response such as to bacterial invasion. M2 macrophages are associated with fibrosis (i.e., extracellular matrix deposition) and tissue repair and regeneration. They also play a role in TH2 immunity. [5], [25], [39], [40]

Macrophages are directly associated with the inflammatory response. Cellular environments such and expression and cytokine presentation play a large role on polarizing macrophages. Roles are varied in both resolving and non-resolving inflammation. Resolving inflammation occurs in the normal healing process in which cells are properly regulated and M1 macrophages turn into M2 macrophages, whereas non-

resolving inflammation has prolonged inflammation such as what occurs in chronic inflammation. [5], [41]

2.1.4 Resolving Inflammation vs Non-resolving Inflammation

During resolving inflammation immune cells are recruited to the site of injury i to return tissue to homeostatic conditions. Monocytes and neutrophils arrive from the blood. Monocytes then differentiate into macrophages, which are then induced into an M1 phenotype. These then release cytokines to help promote repair and regulate healing, however this complex pathway is still not fully understood. When the debris and injury components are cleared up the monocyte-derived macrophages (MDMs) then become more fibrotic. Eventually most MDMs leave the site or die, while some convert properties to become similar to resident tissue macrophages. [5], [42]

Non-resolving inflammation occur in diseases such as cancer, autoimmune diseases, and chronic inflammation and wounds. Monocytes are chronically recruited to the site of inflammation, increasing output of myeloid cells. [43], [44]

2.1.5 Macrophages in Inflammation

Macrophages play a major role in inflammation through their immunological response to remove foreign substances and by rapidly producing cytokines to invoke the inflammatory response. These cytokines have several functions; therefore, macrophages play multiple roles in inflammatory response. Cytokines involved in the inflammatory response include IL-1β, TNF-α, and IFN-γ, while those involved in the anti-inflammatory response include IL4, IL-13, CCL-18, CCL-22, TGF-β and VEGF [45]–[47]. Macrophages also

release chemokines and antimicrobial peptides when activated. Although the inflammatory process is aimed to be beneficial by removing foreign substances and dead cells, it can also cause tissue destruction. When macrophages are exposed to IFN-γ, cytokine production and the inflammatory response is greatly increased, often resulting in an increase in tissue destruction. [48]–[50]

Macrophage expression of cytokines is a complex process and depends on environmental ques such as exposure to pathogen associated molecular patterns (PAMPs) or activation of the kinase-dependent signaling pathway. The kinase-dependent signaling pathway responsible for macrophages activation and cytokine production results in transcription of IFN-γ, NF-κB, CREB and AP-1. Macrophages respond to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) by producing tumor necrosis factor (TNF) [51]. TNF further promotes release of several other inflammatory cytokines including interleukin 6 (IL-6), IL-12/23 and type 1 interferons. Lineage specific receptors which can have differing responses to these cytokines are uniquely represented in macrophages residing in specific tissue types. Macrophages are adept at promoting inflammation in response to LPS due to pattern recognition receptor toll-like receptor 4 (TLR4) and activation via lineage-specific transcription factors including PU.1 and C/EBP [52], [53]. Other transcription factors associated with TLR signaling include signal transducer and activator of transcription (STATs). STAT1, STAT2, STAT4, and STAT5 induce inflammation while STAT3 and STAT6 induce transcription of anti-inflammatory genes. TLR signaling is also mediated by microRNA (miRNA) which are small strands of RNA (21-25 nucleotides in length) that act to suppress gene expression [51],

[54]–[56]. Dysregulation of macrophage miRNAs is seen in disease phenotypes such as cystic fibrosis [57]. [58]

Inflammatory mediators modulate macrophage activity effectively turning off or on inflammation. As such there is much research into inhibition and promotion of these mediators either directly such as with gene silencing or indirectly by affecting another molecule upstream in their pathway. Such mediators include TNF-α, IL-1β, IL-6 and IL-12. TNF-α plays a central role in initiating the inflammatory cascade, and there has been some success targeting TNF-α with antibodies to reduce inflammation [59]–[62]. IL1 α and β are proinflammatory cytokines that affect many tissues and cell types and both signal through IL1 receptor 1. IL-1β is important for homeostatic regulation of sleep, temperature and digestion [63]. It has specifically been targeted due to its implication in pain, inflammation and autoimmune diseases such as rheumatoid arthritis, osteoarthritis, multiple sclerosis arthritis, Alzheimer's disease, and inflammatory bowel disease [64]–[67]. Many drugs target IL-1β or act as an antagonist against its receptors, however it is involved in regulation of many cells and tissue and is released from macrophages as well as many other cell types including mast cells and microglia, so treatments may cause adverse side effects [66]. [58]

Other approaches to decrease inflammation work to generally reduce it (not just one cytokine) such as with nonsteroidal anti-inflammatory drugs (NSAIDs), steroids such as glucocorticoids, kinase inhibitors especially for janus activated kinases (JAKs), spleen tyrosine kinase (SYK) and mitogen-activated protein kinases (MAPKs), and receptor-mediated inhibition to directly inhibit macrophage activation. NSAIDs inhibit

cyclooxygenase (COX) enzymes to reduce prostaglandin inflammation by inhibiting eicosanoid production and has proven helpful for osteoarthritis and cancer [68]–[71]. Glucocorticoids are a group of corticosteroids involved in metabolism of proteins and carbohydrates and is the most common treatment for many chronic inflammatory disease including diseases such as asthma, cancer and COPD [72]. Glucocorticoids reduce several inflammatory cytokines including IL-6, IL-12, TNF-α, and IL-1β [73]. Glucocorticoids can reduce NF-κB and AP-1 activity, protein kinase C, MAPKs, and several downstream inflammatory cytokines [73]–[75]. Glucocorticoids also increase anti-inflammatory transforming growth factor beta (TGF-β) [76]. [58]

2.1.6 Macrophages in Inflammatory Disease

Macrophages play a key role in response to pathogens, immunity and maintaining homeostasis during inflammation. As such they play an important role in managing inflammatory disease. Inflammation is caused by either a biological disorder such as inflammatory bowel disease (IBD), heart disease, stroke, cancer, diabetes, neurological disease, respiratory disease or the body's response to a foreign object or infection. These inflammatory diseases account for approximately 70% of all deaths in the United States, and 63% worldwide according to the CDC [77]. Inflammatory disease is caused by excessive inflammation and macrophages which are responsible for ameliorating the disorder are predominantly stuck in the M1 phenotype. [77]–[79]

Recently there has been much interest in the development of therapeutics which induce an M1 to M2 transition in macrophages [7], [78], [80], [81]. Types of tissue engineering therapeutics include nanoparticle and patch loaded drugs. Wound patches could be helpful especially for skin wounds and may be an ideal application of our study. As inflammatory mediators, macrophages play an important role in healing normal wounds and play a large role in chronic wounds. During normal wound healing, genetic, epigenetic and molecular processes work together [82]. Macrophages direct inflammation, tissue remodeling and repair, and the transition into the proliferative phase of wound healing. However, during chronic wounds tissue is constantly destroyed.

2.2 Skin Wounds

The skin serves as a barrier which acts to protect against physical damage, chemical damage, loss of fluid, thermoregulation, and to prevent infection[83]. Layers of the skin include the epidermis, dermis, and subcutaneous tissue as shown in **Figure 1** [84]. Damage can affect one to all layers of the skin and can cause serious issues even leading to death. Wound healing is a complex process that can be disrupted by many conditions such as age, sex, infection, smoking, obesity, diabetes, vascular disease, and malnutrition [85], [86].

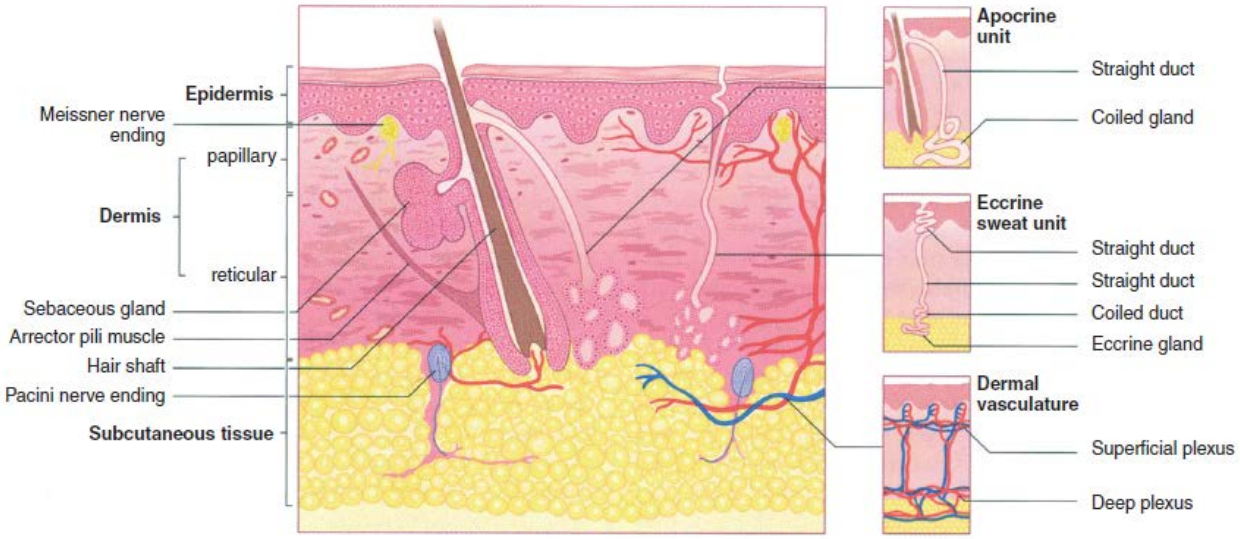


Figure 1. Skin Anatomy

From Andrews' Diseases of the Skin: Clinical Dermatology (10th ed., p.1), by W.D. James, T.G. Berger, and D.M. Elston, 2006, Philadelphia: Elsevier Saunders.

Wound healing is an integral homeostatic process necessary to maintain barrier protection. It is a complex process involving many cells, notably macrophages and fibroblasts. Other hematopoietic and non-hematopoietic cells are recruited including neutrophils, natural killer cells, T cells, B cells, stem cells, epithelial cells and endothelial cells [87]. Macrophages are present throughout the entire wound healing process; however, they play the largest role during the inflammatory phase and proliferation phase. Macrophages digest tissue and cellular debris, regulate inflammation, the process conversion from inflammation to tissue deposition, and support cell proliferation [88], [89]. Inappropriate regulation can lead to excessive inflammation or fibrosis. Macrophages also promote fibroblast proliferation. Fibroblasts are important for tissue

deposition. They produce collagen, glycosaminoglycans, and help to regulate the process by releasing growth factors and cytokines [90]. However efficient the wound healing process is, it does not result in complete regeneration of the tissue and is considered a reparative process. A scar is left behind preventing complete recovery of skin function.[91]

2.2.1 Wound Closure

Wound closure happens in one of three ways, primary, secondary, or delayed primary, depending on the given situation (Figure 2). Primary wound closure, also known as primary intention, occurs when the wound is small and clean. It is often caused by surgical incision or small clean cuts such as paper cuts. Most wounds heal by secondary wound closure. Secondary wound closure, also known as secondary intention, are rougher and require granulation tissue matrix to fill in the defect. It takes longer than primary wound closure and creates more scar tissue. Delayed primary wound closure also known as third intention wound healing is somewhat similar to both primary and secondary wound healing. It occurs when a surgeon opens a wound, cleans it and leaves it for a few days to ensure there is no infection before closing the wound back up to heal via primary intention. This is performed for traumatic injuries when foreign bodies enter the site such as in dog bites and lacerations.[92]

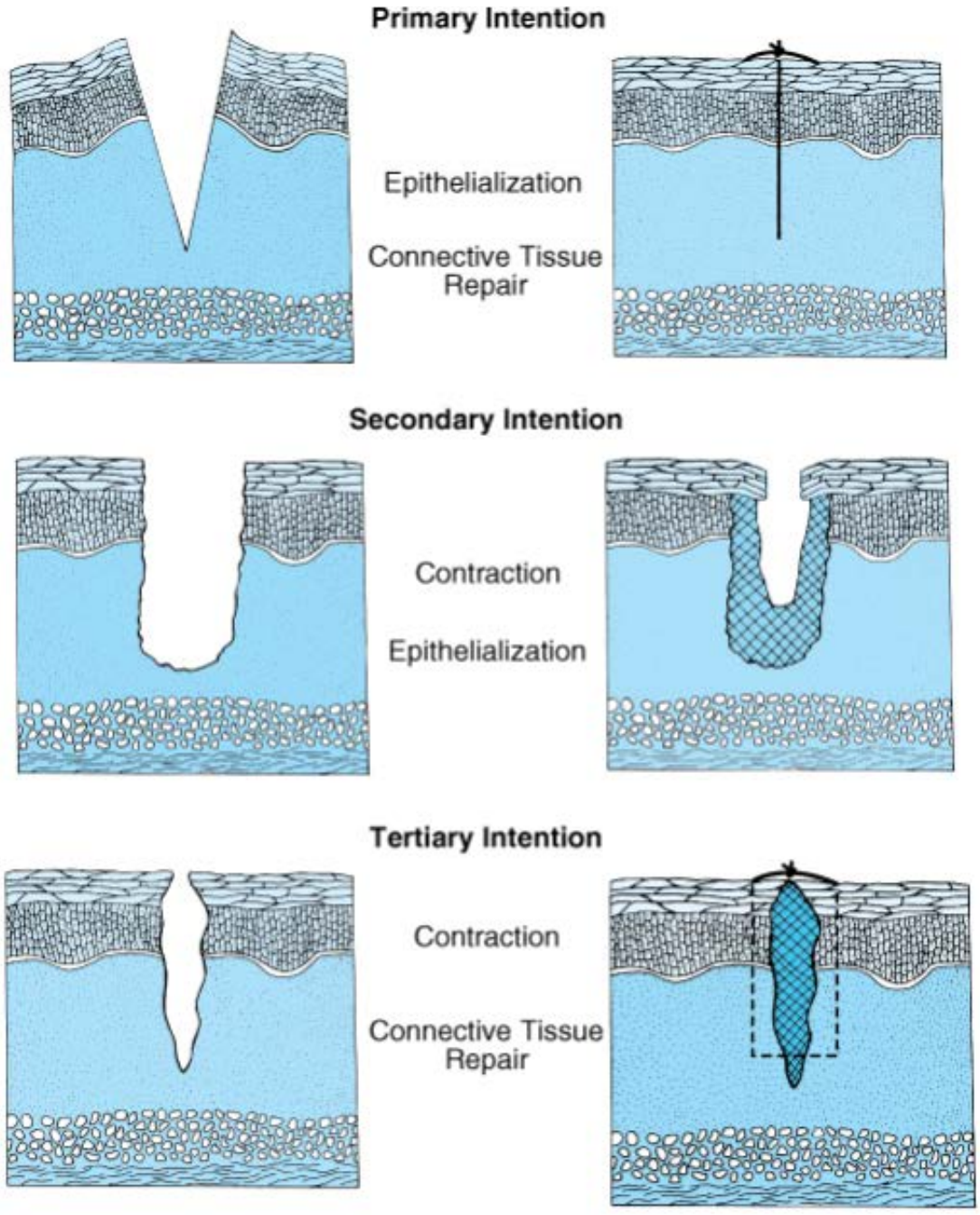


Figure 2. Wound Closure

From Schwartz's Principles of Surgery 9th ed, by Brunicardi FC, Andersen DK, Billiar TR, Dunn DL, Matthews JB, Pollock RE, 2009, McGraw-Hill.

Skin wounds pose a significant problem affecting all people at some time or another. There are many types of open skin wounds including abrasion, laceration, incisions, punctures, avulsion and amputation. Wounds causing damage to the full thickness of the skin including all three layers are the most dangerous, common examples include burn wounds, ulcers and chronic wounds [93].

2.2.2 Types of Wounds

Wounds can be considered either as acute, chronic of complicated.

Acute Wounds

Acute wounds heal themselves normally within 30 days. They are wounds that cannot be healed by primary intention. Acute wounds can occur due to a number or reasons such as biopsies or traumatic injury. Acute wounds can have other injuries associated with them (e.g., broken bone). Both exposed and internal wounds fall into this category [94].

Chronic Wounds

These are wounds that become stalled in one or more phases of the wound healing process, such as the inflammatory phase. Various factors may prolong these stages such as hypoxia, necrosis, infection, and cytokine expression. Causes include pressure, arterial insufficiency, venous insufficiency, burns, age, genetic components and pressure. Continuous inflammation perpetuates a non-healing state. Even when healed, the healed area is mechanically weak and prone to relapse [94].

Complicate Wounds

Complicated wounds are a combination of chronic infection and tissue defect. The cause is due to traumatic injury, such as from an accident or surgery. The wound is contaminated and manifestation of infection depends on virulence, amount of pathogen, blood supply and patient immune system [94].

2.2.3 Phases of Wound Repair

When skin is wounded it goes through four phases to repair the damage.

Phase I: Hemostasis

This phase is the first response to injury primarily to stop the flow of blood. This step occurs within a few minutes of the initial injury. Upon damage host cells release adenosine diphosphage prompting platelets to bind to collagen. Resident macrophages help to regulate clotting [95]. Glycoproteins are released resulting in platelet aggregation. Platelets and the coagulation cascade are activated. Platelets then release vasoconstrictive chemicals locally. Platelets are also responsible for formation of a fibrin clot and initiation of several growth factors including transforming growth factor (TGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). Growth factors then recruit neutrophils, monocytes and fibroblast and stimulate epithelial cells[93], [96].

Phase II: Inflammation

This stage generally lasts up to 4 days. Blood vessels become leaky allowing plasma and neutrophils to enter the site of inflammation. Neutrophils are considered the first line of immunological defense and help to phagocytose and trap infection/debris. Inflammatory cells including monocytes, macrophages, neutrophils and T lymphocytes migrate to the site and remove bacteria, dead cells, damaged tissue and foreign materials via phagocytosis. Monocytes migrate to the site from the blood stream and develop into monocyte derived macrophages (MDMs). Neutrophils have a very short half-life dying quickly which and worsen inflammation, however normally macrophages can help clear their remains. These cells also release more cytokines and growth factors to modulate the healing process and migration/activation of fibroblasts. The site of injury becomes warm and swells due to the influx of cells. Necrotic tissue, extracellular matrix and fibrin is broken down via matrix MMPs to set the stage for tissue deposition. MMPs are secreted by neutrophils, macrophages, epithelial cells and fibroblasts in response to inflammatory cytokines including TNF-α, IL-1, and IL-6 [96].

Chronic wounds and many inflammatory diseases progression of wound healing is stalled at this phase. This may be caused by increased bacterial burden or some underlying disease. [97]

Phase III: Proliferation

In acute wounds proliferation starts at around day 4 and lasts until around day 21. Macrophages, lymphocytes, angiocytes, neurocytes, fibroblasts and keratinocytes work

to deposit extracellular matrix and re-establish skin function. There is angiogenesis, collagen deposition, wound contraction and epithelialization. Macrophages help to regulate this phase by communing with surrounding cells via cytokines. [96]

Phase IV: Remodeling

The remodeling phase constitutes the realignment of collagen to improve the mechanical strength of the tissue. This process can take up to 2 years to finish. Fibroblasts are the main cells involved. Overall cell density decreases in the area while strength is increased. [96]

2.2.4 Chronic Wounds

Chronic wounds include diabetic ulcers, venous leg ulcers, and pressure ulcers and they are defined by ulceration lasting greater than 2 weeks. Wound healing becomes more difficult with age, and chronic wounds become more prevalent in the elderly population [98], [99]. Biofilm-infected cutaneous wounds extend the duration of the wound making treatment more complicated [100]. Chronic wounds become suspended during the inflammatory phase and are commonly accompanied by infection, continued neutrophil accumulation, disordered macrophage polarized in M1 phenotype, disordered lymphocyte function, high levels of proteases, and dysregulation of cytokines/growth factors with inflammatory cytokines being overexpressed [101].

Chronic wounds can be characterized by dysfunctional cytokine expression and growth factor activity [43], [102]. Increased inflammation leads to chronic expression of M1 type macrophages causing unique expression of growth factors and sustained

protease activity such as matrix metalloproteinase 2, 9 and 14 [103]. During the prolonged inflammation phase there are elevated levels of inflammatory cytokines including IL-1α, IL-1β, IL-6 and TNF-α, as well as a large presence of neutrophils [104].

In wounds there is also elevated levels of ROS due to continued presence of neutrophils which release them [105]. Macrophages are able to clear neutrophils during normal wound healing, however since they are stuck in an M1 phenotype they continue to recruit more neutrophils to the wound site further exacerbating the condition [106]. The presence of superoxide contributes to inflammation and tissue destruction [107]–[109].

2.2.4.1 Current Therapies for Chronic Wound Treatment

Treating chronic wounds is a difficult task. A thorough assessment of the wound and patient condition must be described first [110]. Infection poses a particular threat due to the ability of biofilms to evade traditional treatment, they are generally approached with antibiotics and/or tissue debridement [111]. Infection must be treated aggressively. Physicians follow the TIME protocol for wound healing, Tissue assessment, Infection control, Moisture management, Edge of wound management. After management and infection control the wound debridement is an important step to remove the current inflammatory environment and return the wound to more of an acute wound setting. Scalpel, ultrasound and/or enzymes can be used for debridement. The wound is then covered with some sort of wound dressing. Pressure to the wound must be carefully managed especially for pressure associated wounds. Offloading pressure for pressure ulcer healing away from the wound is important, therefore devices such as

braces, casts or specialty shoes may be used in addition to the wound patch. Venous leg ulcers oppositely need compression to help control interstitial fluid build-up. If the wound is not healing, then amputation may be considered [85], [112].

There are several topical therapies that promote wound repair, however their therapeutic effect is limited. Some topical agents include antibiotics, silver, cadexomer iodine, honey, collagenase, saline and hydrogel loaded drugs [113]–[115]. Many dressings are used, such as cotton gauze, hydrocolloid dressings, hydrogel dressings, acrylic dressings, semipermeable film dressings, alginate dressings, hydrofiber dressings, semi-permeable foam dressings, bioactive wound dressings and tissue based products [116]–[118]. If the wound is serious enough, advanced therapies can be considered, such as skin graft or amputation. New therapies have been recently developed which focus on tissue engineering technologies. These include growth factor treatment, acellular skin grafts, skin substitutes and cellular therapies such as with fibroblasts, keratinocytes and/or stem cells. Therapies have also been designed to treat the oxidative stress and inflammatory environment of wounds such as the antioxidant drug catechin ECG which inhibits MMP-2 and MMP-9, or activated protein C (APC) which binds to receptor endothelial protein C receptor (EPCR) to inhibit NFκB and reduce inflammation [104]. [85], [112]

2.3 Electrospinning

Electrospinning is the process by which electrostatic force is used to pull polymers from a liquid solution in the form of fibers. The solution evaporates and only the polymer

is left on the collector. This is an efficient method for producing fibers of nanometer diameter. Electrospinning has been developed by scientist, industrial professionals and entrepreneurs collectively since the early 1900s [119].

2.3.1 Applications of Electrospinning

Electrospinning can be used for many applications in industry and biomedicine, due to its capacity to produce nanofibers. Other processes that can be used to produced nanosized fibers include drawing, which has been discontinued due to limited control of fiber dimension and scalability, self-assembly which is very complex and does not have good scalability or control of nanofiber size/uniformity, phase separation which is limited to specific polymers however again it has poor scalability or control over fiber dimension, and template synthesis which allows for control over fiber dimension but also does not have good scalability. Electrospinning has been used for applications in filters, smart textiles and protective clothing, battery and capacitors, sensors, catalysts, drug delivery, tissue engineering, wound dressing, cosmetics and composite reinforcements, the top three being composite reinforcements, filters and tissue engineering [120]. Electrospinning offers a simple and cost-effective process to provide nanofibers which is both scalable and offers control over nanofiber dimension. [121]

In industry the small fibers form a porous structure, which can be used for filtration, composite materials or as a membrane. Electrospinning is used to create composite fibers allowing multiple polymers to be easily and uniformly combined. Composites allow for altering uses and material properties [122]–[124]. Morphology,

diameter and arrangement of nanofibers can be controlled to suit the application. Electrospinning is commonly used to produce fibers used for tissue engineering scaffolds such as wound patches and scaffolds for tissue regeneration [125], [126]. Polymer properties must be optimized for the nanofiber application such as porosity, biocompatibility and tensile strength. Electrospinning of tissue engineered scaffolds can incorporate molecules, proteins and even live cells [127]–[130]. Fibers can also be aligned for applications such as nerve-tissue engineering [131]–[134]. Nanofiber scaffolds can be modified to optimal pore size for drug loading in the scaffold. Drugs can either be electrospun with the polymers or loaded afterwards. [121]

There are several polymer and solvent combinations that can be used for electrospinning. A few common polymers include PLA, PCL, PLGA, PEG, PEO, and collagen. A few common solvents include Water, DMF, DCM, Chloroform, ethanol, and THF. Proteins like bovine serum albumin (BSA), DNA, RNA and growth factors can be incorporated into the polymer solutions and electrospun into the fibers as well. Different drugs can be loaded into electrospun scaffolds, including ibuprofen, ketoprofen, mefoxin, doxorubicin hydrochloride, fenbufen, paclitaxel, and dichloroacetate [135]–[140], [141], [142].

2.3.2 Process

In electrospinning an electric field polarizes the solvent and causes electrostatic repulsion from the needle and attraction of the oppositely charged plate. There are many setups, one of the most common involving a horizontal plate and syringe parallel to the ground.

This setup will be discussed and is depicted in Figure 3. This setup consists of a single horizontal plate and syringe placed parallel to the ground. There are many other methods of performing electrospinning. Other common versions include a rotating drum to align nanofibers, and also a vertical plate set up in which gravity has more of an effect of the spinning and Taylor cone formation [143]–[145].

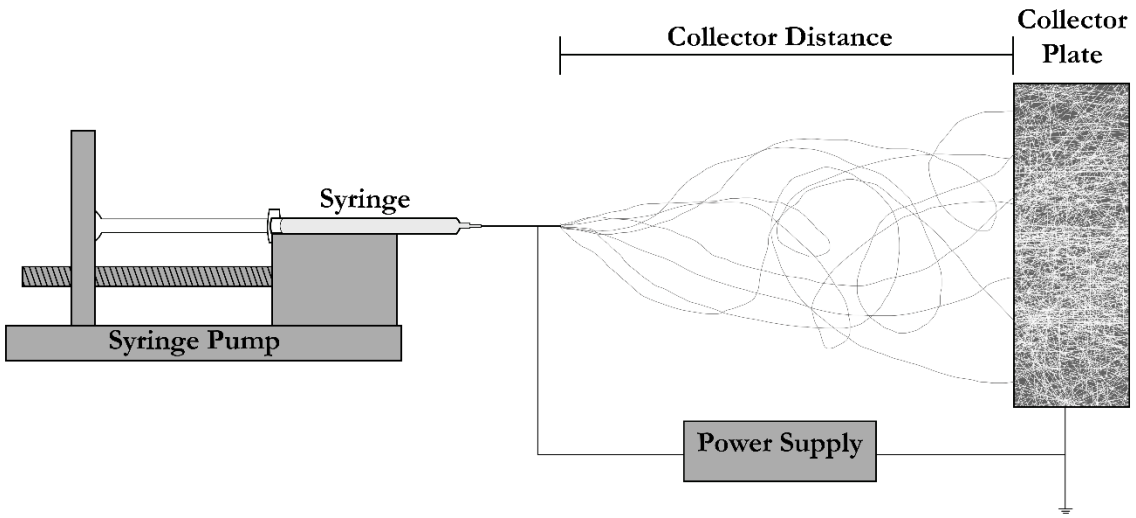


Figure 3. Electrospinning Process

Polymers are first dissolved in the solvent of choice. The solvent can affect the properties of the nanofibers so appropriate solvent and polymers must be chosen. Drugs or other materials can be added at this time to the solution. The solution is then loaded into a syringe and a blunt needle is attached. Needle diameter is one of the factors that can be adjusted to improve nanofiber diameter. Environmental conditions can be adjusted based on solution components (e.g., light sensitivity or temperature). The

distance between the collector plate and the needle is one of the parameters affecting the nanofiber diameter. The flow rate setting on the syringe pump also affects the nanofiber diameter. A faster flow often yields a smaller diameter. The positive electrode is connected to the needle tip and the negative to the collector plate. The collector plate should be made of a conductive material. When everything is set, the syringe pump and power supply can be turned on. A small Taylor cone should appear, and a line should be visible coming from it and going towards the collector plate. The power supply voltage can be modified to adjust material properties. Results depend on the polymers and solvent being used. As weight percentage of the polymer increases in solution the critical voltage for fiber formation also increases. Electrospun nanofibers are often characterized by SEM and FTIR to confirm nanofiber morphology, diameter and composition. If beading occurs during the fiber formation, the flow rate should be increased to make sure the needle and collector are making continuous contact. If fibers form ribbons, a higher polymer concentration or more volatile solvent should be used. To make the nanofibers more porous, a more rapidly evaporating solvent should be used, and for smaller pores a less rapidly evaporating solvent can be used [146]–[149].

2.4 Polymer Comparison

The intention behind this project is to develop a wound patch for healing chronic wounds. Therefore, we decided to work on converting M1 macrophages into M2 macrophages. To this end several polymers were initially compared in **Table 1**. Pullulan

was shown to be relatively inexpensive, with good mechanical strength, easily modifiable, capable of quenching reactive oxygen species (ROS) and promoting neovascularization. Reducing ROS should help reduce inflammation and neovascularization is an important step in wound healing. Compared to other polymers, this seemed the better for wound healing. Gelatin was added to improve cellular adhesion and due to its ability to support wound healing by attracting fibroblasts and macrophages. Ethylene Glycol Diglycidyl Ether was chosen as the chemical crosslinker between the polymeric fibers due to its biocompatibility and proven ability to crosslink both polymers [150], [151], [28], [29], [152], [153].

Criteria	Pullulan [28], [154]-[158]	Gelatin [155], [159]-[161]	Chitosan [160], [162]-[164]	Elastin [165]	ELP [161],[166]
Source	Aureobasidium pullulans	Connective Tissue	Natural- from shrimp shells	Natural- ex. bovine neck tendon	Natural- ex tropoelastin
Degradation	50% in 20 min	25% after 1 day, 3-30% after 1 day	5-15% after 1 week in vivo	Doesn't degrade quickly	
Potential Crosslinker	EGDE, Cysteamine, trisodium trimetaphosphate, (1,4-butanediol?)	EGDE, Glutaraldehyde, photo crosslinking, PEG, 1,4-butanediol	photo crosslinking, Hyaluronic Acid	Glutaraldehyde	
Biocompatibility	Good, reduces ROS, promotes neovascularization	Good. Promotes clotting and ECM formation. Attracts fibroblasts and macrophages	Good		
Source	polysaccharide	Protein	Polysaccharide	Protein	Peptide of tropoelastin
Composition	Composed of α 1,6 linked maltotriose units (G3), each with 2 α 1,4 glycosydic bonds	Protein, 3 α helical peptide chains to form a triple helix. Hi content of hydroxylated amino acids	β-(1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. Basic. Soluble in acidic pH	Composed of simple amino acids. Made by linking tropoelastin protein molecules	Composed of sequence $[VPGXG]_n$ where x represents a common amino acid except proline.
Alterability	Good, 9 easily modified OH bonds	Good, OH bonds	Primary amines. High charge density.		Altering peptide repeats and amino acid
Additional Features	high water absorption, good mech. properties	Good cellular attachment	Forms films, antimicrobial, anti inflammatory		Nonimmunogenic, good for drug delivery

Criteria	Fibrin [159], [167]	Alginate [168], [169]	HA [170]-[172]	PEG [173]-[175]
Source	Blood Plasma	Brown Algae	Extracellular Matrix	Synthetic
Degradation	2days-1month	Biodegradable	66% in 30min, sHA1-AC took 24-48 hours	Varys depending on composition
Potential Crosslinker	Thrombin, calcium chloride	ADA, 1-ethyl-(3-dimethylaminopropyl) carbadiimide hydrochloride	Photo-crosslinking	Photo-crosslinking
Biocompatibility	Good, supports hemostasis, inflammatory cell recruitment, angiogenesis	Good	Good, facilitates structure during early wound healing, promotes keratinocyte activity	Good
Source	Protein	Synthetic/natural hybrid	Synthetic/natural hybrid	Biodegradable synthetic
Chemical Composition	Fibrinogen and thrombin	Anionic polysaccharide	Non-sulfated glycosaminoglycan (GAG)	Polyether with common structure. Available in multiple geometries
Alterability			Binding with benzyl alcohol makes HA more alterable	Highly alterable
Additional Features	Prevents wound contraction[15]	Gels at skin temperature, pro inflammatory, high levels of endotoxins, component of biofilms of *P. aeruginosa*	Polysaccharide common to most species, weakens ECM and permits cell migration, anionic	Nonionic

Table 1. Polymer Comparison.

Polymers compared are: pullulan, gelatin, chitosan, elastin, elastin like peptide (ELP), fibrin, alginate, hyaluronic Acid (HA), and poly ethylene glycol (PEG). Properties examined were: degradation, crosslinker, biocompatibility, source, chemical composition, alterability and any additional feature that promote biomedical applications

2.5 Cross-linking

Cross-linking is the process of chemically bonding a polymer or multiple polymers together and is usually done to increase the mechanical properties of the material. Electrospun nanofibers used in medical application are often crosslinked to prevent immediate degradation in moist environments [176]–[179]. Stability of the crosslinked product is dependent on the type of polymer, type of cross-linking agent, concentrations and environmental conditions, such as temperature, play a large role in the chemical reaction which produce the cure effect on the final product [180]. Stability of the polymer post-crosslinking depends on the degree of crosslinking, the stability of the crosslinker and of the polymer/polymers used, and environmental conditions (i.e., pH, temperature, mechanical trauma, etc.). Crosslinkers generally have two or more reactive groups and react with functional groups such as hydroxyl, carboxyl, amine and sulfhydryl. Crosslinkers

can be specific or less so in the case of photoreactive crosslinking. Crosslinking is used for scaffold strengthening and material immobilization (e.g., drugs or biomolecules) [181].

Common crosslinkers include those containing functional groups such as maleimide, sulfhydryl and succinimidyl esters. Sulfosuccinimidyl esters are water-soluble crosslinkers and are useful for crosslinking when organic solvents should not be used. The chemical structure of the polymer is altered due to crosslinking. This can change the polymers functional properties. Crosslinkers can form polymers from monomers, covalent bonds between polymers or ionic bonds between polymers. There are also cleavable crosslinkers like sulfoxides. Energy can be added to stimulate the reaction via heat or pressure. High-energy ionizing radiation such as from gamma radiation, x-ray, or electron beam can also be used to crosslink material. [182]

Crosslinkers can be used to create polymers from monomers, to connect multiple polymers, proteins, or larger structures. There are physical crosslinkers and chemical crosslinkers. Physical crosslinking occurs by ionic interaction, crystallization, protein interaction, hydrogen bonds, steric complex formation, or hydrophobic interactions. Chemical crosslinkers synthesize polymer growth or polymer bonding. Chemical crosslinkers include homobifunctional crosslinkers and heterobifunctional crosslinkers. Homobifunctional crosslinkers have identical reactive groups and are used to affix certain functional groups they react with. Heterobifunctional crosslinkers have different reactive groups allowing conjugation of molecules with dissimilar reactive groups. Example crosslinkers include disuccinimidyl suberate (DSS), ethylene glycol diglycidyl ether (EGDE), glyoxal, silane, glutaraldehyde, and ethylene glycol dimethacrylate [183]–[190]. These

crosslinkers can be combined with polymers such as poly(vinyl alcohol) (PVA), poly(ethylene glycol), proteins, gelatin, chitosan, cellulose, polyacrylamide, and alginate [191]–[197]. Hydrogels are an example of crosslinked polymers that are often used in the biomedical industry. Electrospun fibers can also be used as hydrogels for wound dressing and other purposes [198]–[200]. Polymers and crosslinkers can be used in a variety of industries such as packaging, adhesives, textiles, food, drug delivery and tissue engineering. [182]

Hydrogels are macromolecule gels constructed from chemically crosslinked polymer chains. They are synthesized from monomer crosslinking or by crosslinking of a polymer (e.g., crosslinking of electrospun nanofibers). Hydrogels can provide ideal conditions for drug delivery and as a scaffold for tissue engineering [201]–[206]. [182]

2.5.1 Ethelene Glycol Diglycidyl Ether (EGDE)

Ethylene Glycol Diglycidyl ether (EGDE) is a common crosslinking compound and has been used for crosslinking polysaccharides, proteins and organic molecules such as chitosan and gelatin [207]–[210]. Crosslinking with EGDE improves water resistance and mechanical properties of its target. EGDE has been effective for crosslinking gelatin pullulan, chitosan, lignin, DNA and PVA as reported by several authors [150], [211]–[216]. EGDE has been shown to have better biocompatibility than the common crosslinker glutaraldehyde [217]. EGDE contains two highly reactive epoxide functional groups at either end of the molecule. The three-member ring is a cyclical ether that is very reactive due to strained covalent bonds. This allows EGDE to bind with hydroxyl, carboxylic and

amine functional groups (**Figure 4**) [218]. Temperature and pH may be adjusted to improve crosslinking [215]. EGDE is able to react at a wide range of pHs, however citric acid is commonly used to catalyze the reaction [150]. [151], [218]

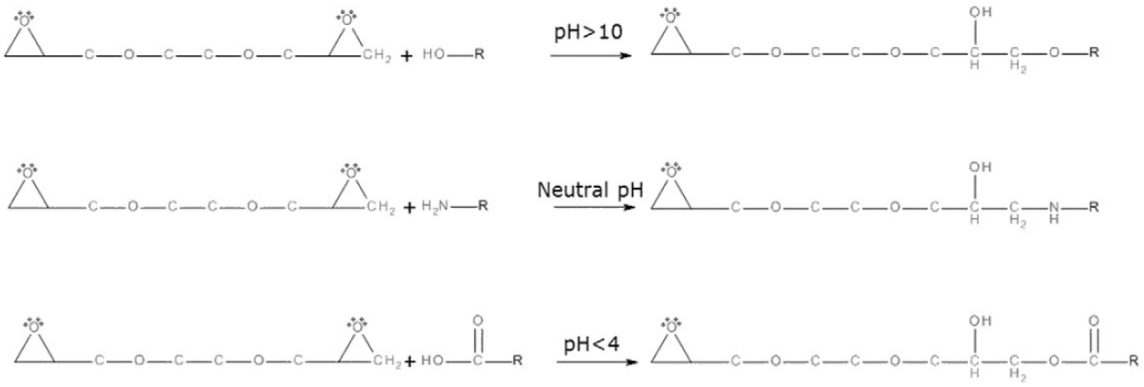


Figure 4: Crosslinking Reaction of EGDE with Hydroxyl, Amine and Carboxyl Groups (*Poursamar et al, 2016*) [220]

2.6 Pullulan

Pullulan is a polymeric exopolysaccharide commonly extracted from the yeast like fungus *Aureobasidium pullulans*. It has been gaining traction for use in tissue engineering due to its high biocompatibility and tissue regenerative properties. Pullulan is non-hygroscopic, dissolves readily in water and is an FDA approved food additive that is slow digesting and low in calories making it ideal for food preservation. Due to its lack of

functional groups promoting cell binding, it should form a copolymer with proteins providing such binding motifs, such as gelatin [28], [29], [219]. [152]

2.6.1 Properties of Pullulan

Pullulan is a linear, unbranching, amphiphilic molecule composed of 9 hydroxyl groups making it easily modifiable and crosslinkable such as with EGDE. It is composed of repeating α (1-4) maltotriosyl units (3-D-glucopyranosyl) with adjoining α(1-6)bonds [220]. Pullulan can reduce reactive oxygen species which can improve the inflammatory environment in wounds [15]. It has good mechanical properties, hydrophilic, hemocompatible and has good swelling ability. [155]

2.6.2 Pullulan Production

Pullulan is industrially produced by fermenting liquified starch from a source such as sucrose, glucose, soy bean oil, beet molasses and/or coconut byproduct with *A. pullulans* [221]–[223]. Besides being produced by *Aureobasidium pullulans* , other yeast and fungi also produce pullulan including *Cytaria darwinii, Teloschistes flavicans, Rhodotorula bacarum and Cryphonectria parasitica* [224]–[226], [26].

2.6.3 Pullulan Application

Pullulan has proven to be an ideal polymer for applications in vascular tissue engineering, cartilage repair, bone tissue engineering and wound healing [227]–[231]. It is modifiable to form carboxymethyl pullulan and sulfated pullulan [232], [233]. Pullulan

has been shown to improve osteoconductivity and provide mechanical stability for bone and tooth repair [230]. Pullulan is an ideal polymer for wound healing due to its ability to protect the wound from bacterial infection, its modifiability, and ability to maintain a moist environment and prevent fluid loss [157], [234]–[236]. Pullulan has been used for drug delivery and antibiotics can be loaded into pullulan without loss in bioactivity [237]–[239]. Pullulan can be loaded with many cell types including mesenchymal stem cells, macrophages, smooth muscle cells, and human endothelial cells [156], [227], [240]. Pullulan is shown to be effective to improve wound healing by including a copolymer such as gelatin and/or by incorporating of mesenchymal stem cells [154], [156].

2.7 Gelatin

Gelatin is a biopolymer derived from the hydrolysis of the protein collagen. Both collagen and gelatin are commonly used in tissue engineering. There are benefits to both. Both have great biocompatibility, gelatin is inexpensive and easier to work with however it has worst mechanical properties than collagen. Gelatin is commonly used in foods, cosmetics, pharmaceuticals and for biomedical applications. It is especially well known for its good cellular adhesion which is why it is often used as a copolymer for wound healing applications [241].

2.7.1 Properties of Gelatin

Gelatin is a polypeptide containing 18 amino acids with large amounts of glycine, proline, alanine and hydroxyproline. Gelatin is water soluble however it requires initial

heating to at least 35°C. Alkali processed gelatin has predominantly alanine while acid processed gelatin is predominantly glycine. Gelatin structure is linear and characterized by hydroxyl groups, carboxylic groups and amino groups. Some properties such as peptide chain size and organization of bonds can be altered by the manufacturing process. [242]

2.7.2 Gelatin Production

Gelatin is derived from skin and connective tissue. Commercially, gelatin is made from cattle bones, animal skins, and fish skin [243]. Depending on the source, gelatin is extracted by alkaline pretreatment or acid pretreatment resulting in gelatin with different properties. These processes affect the isoelectric point, pH and other properties. Gelatin type A (GA) which is acid pretreated has an isoelectric point of 8-9 and is positively charged at neutral pH, and gelatin type B which is alkaline pretreated has an isoelectric point of 4.8-5.4 and is negatively charged at neutral pH [244]. We used Gelatin Type A for our experiments.

2.7.3 Gelatin Application

Gelatin is use in food due primarily to its gelling and thickening properties and is used as a stabilizer in yogurt, and thickener in jam [245]. It is commonly used in tissue engineering for application in drug delivery, wound healing and tissue regeneration [153], [246], [247]. It is being used as a drug carrier of anticancer drugs such as curcumin, paclitaxel, and doxorubicin (DOX), it has been used in polymer composites to improve bone and skin regeneration, and it has been shown to act as an ideal wound healing

scaffold component for hydrogels and has been used with other cell types such as mesenchymal stem cells and fibroblasts in biomimetic scaffolds to facilitate healing [27], [248]–[258].

2.8 Retinoic Acid in Wound Healing

Retinoic acid (RA) is a metabolite of vitamin A, and the most biologically active intermediate in the retinol metabolic pathway [259]. It can be presented in multiple isoforms such as the 13-cic-RA isoform, however, the all-trans isoform is the most common in tissue and will be the focus here. RA is produced *in vivo* as a signaling molecule for embryonic development and is known to play a role in axial patterning, neurogenesis, limb and organ development, and lymphoid development [260]. It is not entirely sure whether RA can be delivered between cells or that its' production relies on stimulation through other pathways. RA transcriptionally regulates gene expression by binding to retinoid receptors such as RAR and RXR which are common in most cells. The effect of activating retinoid receptors differ depending on the cell type [261], [262].

In stem cells, when RA is produced it is transported via CRABP2 to the nucleus. In the nucleus RA binds to an RAR receptor. RAR then binds to an RXR receptor forming a heterodimer which binds to DNA and activates transcription. Many genes are both directly and indirectly regulated as a result. RAR and RXR need to be phosphorylated to actively bind and react to RA. Co-activators and co-repressors can bind to the RAR/RXR heterodimer to modulate this response [263].

RA has been shown to decrease inflammation and regulate macrophages and mesenchymal stem cells and to promote wound healing. RA is used therapeutically to reduce injury and fibrosis in acute kidney injury, with studies showing M1 inflammatory macrophages become alternatively activated to M2 phenotype [264]. Pourjafar, et al., showed that pretreating MSCs with all-trans RA improves MSC viability and activity, and enhances overall proliferation and angiogenesis in a rat incision wound model [13]. In another study Abdelhamid, et al., showed that after exposing MSCs and peripheral blood mononuclear cells (PBMCs) to lipopolysaccharide (LPS) which stimulates an inflammation, treatment with RA improved viability and reduced the inflammatory response [265]. RA has also been shown to effectively convert M1 macrophages into M2 phenotype [266]. Lin, et al., showed that RA can stimulate M1 to M2 conversion, reduce inflammation and significantly improve wound healing; furthermore they found that M2 macrophages treated with RA causes activation of Arg1 which is a crucial gene for wound healing [267]. Overall RA decreases inflammation, improve angiogenesis, convert M1 macrophages to M2 macrophages and improve wound healing making it a novel therapeutic for inflammatory diseases such as chronic wounds.

2.9 Mesenchymal Stem Cells in Wound Healing

Mesenchymal stem cells (MSCs) have the ability to renew themselves and to differentiate into many cell types including adipocytes, chondrocytes, osteoblasts, myoblasts, fibroblasts and chondroblasts [268]. MSCs can be found in multiple locations

in the human body including bone marrow, adipose tissue, synovial tissue, and lung tissue. They are often isolated from bone marrow (bone marrow derived mesenchymal stem cells = BMSCs) or adipose tissue (adipose derived mesenchymal stem cells = ADMSCs) for research purposes. Adipose derived stem cells are particularly useful due to their ease of harvest and accessibility. MSCs help to maintain homeostasis and play a role in wound healing. MSC treatment has the potential for regeneration due to its ability for reverse remodeling, cell regulation and to differentiate into important cells at the wound site. They can be identified by the surface markers CD73, CD90 and CD105. MSCs have been shown to suppress inflammation from neutrophils, macrophages, dendritic cells, natural killer cells, mast cells and eosinophils [269].

In cutaneous wounds MSCs use paracrine signaling to increase angiogenesis, regulate inflammation and ECM, and enhance epithelialization and wound closure [270]. MSCs can signal fibroblast and keratinocytes to migrate to the wound site [271]. They are also able to inhibit the expression of MMPs which degrade tissue, thereby paving the way for extracellular matrix deposition [23], [272]. Many studies have shown that MSCs are able to improve chronic inflammatory disease and accelerate wound closure of chronic wounds [16], [28], [273]–[278]. Rustad, et al., showed that pullulan and collagen hydrogels loaded with MSCs induce MSCs to secrete angiogenic cytokines, promote pluripotency and to promote stemness factors Oct4, Sox2 and Klf4 [154]. Wound microenvironments can be harsh due to reactive oxygen species, inflammatory cytokines and cytotoxic mediators. Kosaraju, et al., showed that ADMSCs seeded on pullulan-collagen hydrogel enhances survival of ADMSCs in the wound environment and promote

recruitment of circulating BMSCs [156]. Chen, et al., found that pullulan/collagen + MSC hydrogels could inhibit M1 macrophage expression, promote secretion of TFG-β1 and bFGF (known to regulate keratinocytes, fibroblasts and endothelial cells), and improved wound closure [279]. In conclusion, MSCs can improve healing and regeneration in chronic inflammatory diseases such as chronic wounds. In chronic wounds, MSCs, pullulan, collagen/gelatin and RA can further improve wound healing via increased angiogenesis, increased MSC stemness, decreased ROS, decreased MMPs and conversion of macrophages from M1 to M2 anti-inflammatory phenotype.

CHAPTER III

Materials and Methods

3.1 Electrospinning

The materials used for electrospinning were Concentrations of 200,000MW pullulan (Hayashibara Laboratories, Okayama, Japan) and gelatin Type A gelatin from porcine skin (Electron Microscopy Sciences, Hatfield, PA). The polymers were first dissolved in water at 20wt%. Polymer concentrations electrospun were 100% pullulan, 75% pullulan/ 25% gelatin, and 50% pullulan/ 50% gelatin. Solutions were heated to 50^{O}C and magnetically stirred for 30min to make the polymers go into solution. The solution was then sonicated for 30min to get rid of bubbles. The polymer solutions were then loaded into 10mL BD Falcon syringes with an attached 22-gague needle attached. The sample was then loaded onto a syringe pump. The electrospinning process was carried out at 50^{o}C by using a heat gun to maintain liquid phase of the solution. The device setup consisted of a syringe pump, a high voltage power supply (Information Unlimited, Inc.), and a collector plate

covered with non-stick aluminum foil. Pullulan/gelatin composite solutions were loaded in 10mL syringes and placed horizontally. The solution was expunged through a 22-gague blunt-end needle and fibers were collected. The high voltage power supply was set to 37kV, the flow rate was set to 55µL/min (3ml/hr), and the distance between syringe needle and collector plate was set to 18cm. The scaffolds were stored at 4°C in a vacuum desiccator until analysis or crosslinking could be performed.

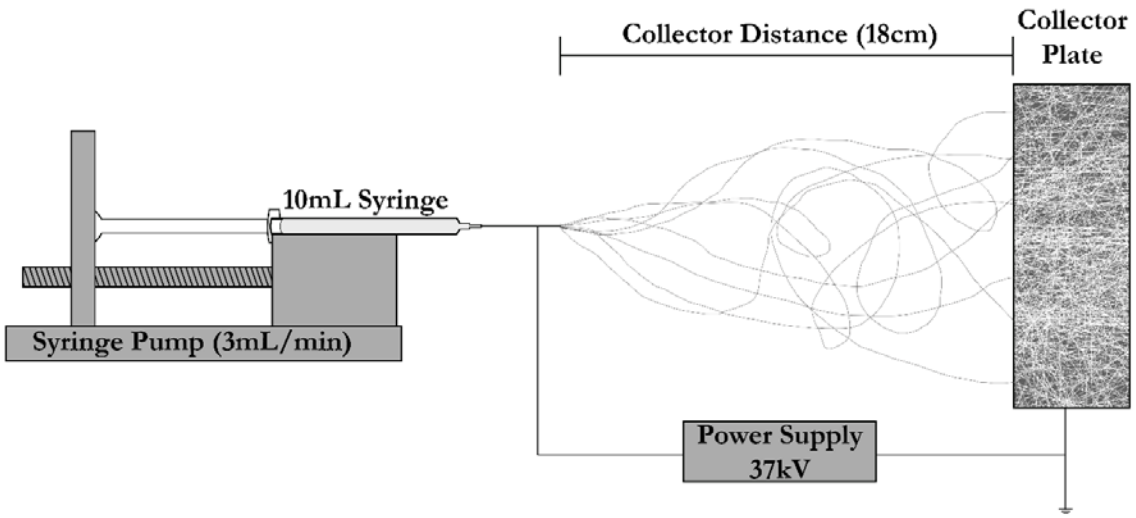


Figure 5. Pullulan/Gelatin Electrospinning Setup

3.2 Crosslinking

Crosslinking solutions were prepared at different ratios of EGDE to absolute ethanol. Both pullulan and gelatin are known to be crosslinked by EGDE. Ratios tested were 1:100, 1:50 and 1:70 with the addition of 0.05M citric acid. Pullulan/gelatin nanofibers were

immersed in crosslinking solution for 24h to crosslink. Crosslinked nanofibers were dried at 50°C for 24h. This is a modified protocol based on a method described by Li, et al., to crosslink gelatin [150].

3.3 Scaffold Loading

After crosslinking with EGDE, nanofiber scaffolds were loaded with RA. Nanofiber scaffolds were first rinsed with PBS to remove any residue from EGDE crosslinker and then sterilized with UV for 1h prior to loading to prevent contamination. RA was diluting in EtOH, syringe filtered, and added to the sterilized scaffold, then dried. The amount or RA added was 5µg or 10µg per 10µg scaffold (this is equivalent to 5mg/mL or 10mg/mL RA in cell culture media respectively). Passage 4 ADMSCs were also loaded onto the scaffold. ADMSCs were incubated with the sterilized scaffold for 3 min. If both RA and ADMSCs were added to the same scaffold, RA was added first, dired, then the ADMSCs were added.

3.4 Cell Culture

Human Monocytic THP-1 cells (ATCC® TIB-202™, Manassas, VA) were maintained at Wright State University in RPMI culture medium (RPMI 1640, Life Technology, Grand Island, NY) containing 10 % heat inactivated FBS (GE Hyclone, Marlborough, MA). THP-1 cells were differentiated into macrophages by incubating 24h with 200nM PMA (Cayman

Chemicals, Ann Arbor, MI) and supplemented with 30ng/mL GMCSF (GenScript, Piscataway, NJ) for macrophage growth. Using PMA is a commonly used method to differentiate THP1 monocytic type cells into macrophages [280]–[283], [284, p.], [285, p. 1]. Macrophages were polarized to M1 macrophages by incubation with 100ng/mL LPS (eBioscience, San Diego, CA) and 100ng/ml IFN-γ (Invitrogen, Rockford, IL) and incubated at 37°C, 5% CO_2 for 24h. Adult non-diabetic ADMSCs from Lonza were cultured to passage 4 in MSC basal medium (ScienCell, Carlsbad, CA). In co-culture experiments, THP-1 cells were first differentiated in 24 well plates. After polarization, cells were washed and resuspended in 1mL 10 % FBS with RPMI. ADMSCs were cultured from passage 3 until confluent. They were then split and concentrated to 1 million cells per 10µL. 10µL of ADMSCs were then added to the scaffold. They were incubated on the sterile electrospun scaffold for 3 minutes, then loaded into transwell inserts (Corning, New York, NY).

3.5 Reagents

200nM PMA was used for macrophage differentiation. Macrophages were differentiated in RPMI, FBS and GMCSF. Macrophages were differentiated for 24h then macrophages were polarized. IFN-γ was used at a concentration of 100ηg/ml and LPS was used at a concentration of 100ηg/ml for macrophage polarization to M1 type macrophages. Macrophages were polarized to M1 phenotype for 24 hours then cells were washed and treated. Treatments include RA and PGG. RA (Sigma Aldrich, Saint Louis, MO) was dissolved in DMSO at 50mg/mL. Treatment of RA in culture was at 5µg/mL and 10µg/mL.

3.6 Scanning Electron Microscopy (SEM)

SEM images of nanofiber scaffold were obtained using a Phenom Pro scanning electron microscope. Electrospun nanofibers were sputter coated with 10nm of iridium. Scaffolds concentrations imaged were 100% pullulan, 75% pullulan/ 25% gelatin, and 50% pullulan/ 50% gelatin. Scaffolds were imaged with and without crosslinking with EGDE. Images are representative of the average. Nanofiber diameters were measured using ImageJ software. Diameter averages were compared statistically using JMP software. 3 images were taken for each sample with 20 nanofiber diameters measured for each image.

3.7 Fourier-Transform Infrared Spectroscopy (FTIR)

Scaffold compositions were determined by loading onto an attenuated total reflectance (ATR) attachment and using a Thermo Scientific Nicolet iS 50 FTIR (Thermo Fisher, Waltham, MA). Scaffolds were tested with and without RA. Data was plotted in Excel.

3.8 Scaffold Degradation and Drug Release

For degradation the scaffold was crosslinked at either 1:70 or 1:50 EGDE in EtOH. After crosslinking in EGDE RA dissolved in EtOH was added at either 5µg or 10µg and then allowed to dry. The scaffold was incubated with 1mL Ringers solution in 37^{o}C incubator with shaking at 100rpm. Samples for each concentration was measured in quadruplicate.

The same sample was used consistently for both drug release and degradation. Ringers solution was collected and analyzed for RA using a spectrophotometer at 316nm. Time points for drug release collection was 1h, 4h, 8h, 24h, 72h, 168h. Time points for scaffold degradation was 1day, 3days, 7days, 14 days. For time points that coincide between drug release and degradation, Ringers solution was collected then scaffold was dried at 50^{o}C for 24h and the weight of the contents measured. 1mL of fresh Ringers solution was then added and incubation was resumed. RA concentration was measure using a spectrophotometer at 316nm wavelength. Statistics for RA release and scaffold degradation were measured using two-way ANOVA.

3.9 Live/Dead Imaging

An Invitrogen ethidium/calcein kit (Invitrogen, Rockford, IL) was used to stain for live and dead cells. Ethidium penetrates damaged cell while Calcein is retained only by living cells. Live and dead cells were imaged using a Leica fluorescent microscope. cells have emission - excitation wavelengths of 495nm - 515nm for live cells, 528nm - 617nm for dead cells.

3.10 qPCR

M1 to M2 macrophage polarization for treatment with RA and MSC loaded electrospun and EGDE crosslinked pullulan/gelatin scaffold using qPCR. M1and M2 markers were assessed. M1 markers: TNF-α (Sinobiological, Beijing, China), IL1β (Sinobiological, Beijing,

China). M2 markers: CCL22 (Biomol, Pompano Beach, FL), CCL18 (GeneCopoeia, Rockville, MD). Macrophages were plated at 2e6 cells per well. 10µg scaffold was added to each transwell with RA loaded at 5µg or 10µg and/or passage 4 MSCs loaded at 1e6 cell. Cells were incubated for 2 days with treatment and then M1 and M2 markers expression was assessed. After incubation cells were washed with PBS. Trizol reagent (Invitrogen, Rockford, IL) was used to isolate RNA. NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA) was used to measure RNA concentration. Approximately 300ηg of RNA was transcribed into cDNA using Applied Biosystems high-capacity cDNA reverse transcription kit (Foster City, CA). SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) was used for qPCR setup, and StepOnePlus real time qPCR machine from Applied Biosystems (Applied Biosystems, Foster City, CA) performed reaction and analysis. Amplification reaction Setup: 95^{o}C 5min for pre-denaturation, 50 cycles of [95 ^{o}C 20s, 60 ^{o}C 40s, 72 ^{o}C 20s], Melt curve. GAPDH was used as control to determine relative gene expression. fold change was calculate using the formula: fold change = 2^- ΔΔCt.

3.11 Statistical Analysis

All data was compiled in Excel then transferred to JMP student edition 10 statistical software from SAS. JMP was used to perform t tests for nanofiber diameter and all qPCR assays. JMP was also used to perform two-way ANOVA on Scaffold Degradation and RA release rate.

CHAPTER IV

Results

4.1 Scaffold Composition

Pullulan is a linear, nonionic, water soluble exopolysaccharide composed of α-1,6-linked maltotiose residues and produced by yeast like fungus *Aureobasidium pullulans*. It is composed of 9 hydroxyl groups making it a highly modifiable compound. Pullulan is useful in biomedicine because it is amenable to manipulation, non-toxic, biocompatible, blood compatible, non-toxic, biodegradable has antioxidant properties. It also has other properties such as being good mechanical strength and non-reducing and thermal stability. Pullulan has been FDA approved for use as a food preservative due to its slow digestibility and low-calorie count. Recent evidence supports pullulan as an ideal therapeutic target for tissue engineering and wound healing [152], [219].

Pullulan and gelatin scaffolds have shown promising wound healing abilities. Gelatin is a polypeptide derived from the hydrolysis of the collagen protein. Collagen is an important component of the extracellular matrix of connective tissue. It is ideal for use

in tissue engineering in scaffolds however it is not as easy to work with a gelatin. Gelatin is a good alternative to collagen, the major downside is that it loses some of its mechanical properties during the denaturing process, however crosslinking can improve its mechanical properties. Gelatin is also less immunogenic than collagen. The polypeptide arrangement of Gelatin provides a RGD motif which causes cellular attachment to the gelatin and can be very useful in scaffolds. Gelatin can also signal differentiation, and proliferation. Gelatin is FDA approved for use as a food additive and gelling agent as well as recently for tissue engineering applications such as drug delivery, wound healing, regeneration, as well as the food industry [27], [153], [241], [242], [257].

Pullulan and gelatin seem to be a good pair for wound repair, offering ROS quenching and cellular attachment as well as being a potential vehicle for drug and mesenchymal stem cell delivery. Therefore, we chose to use pullulan and gelatin as the base for our scaffold. Previous studies have shown that pullulan and gelatin can improve wound repair, and that cells such as MSCs can be loaded onto such a scaffold to promote improved wound healing[15], [28], [152], [153]. We decided to electrospin pullulan and gelatin which is an easy quick, cost effective and scalable method of creating scaffolds that allows tailorable nanofiber diameter size and porosity [141], [286]. This is the first-time gelatin/pullulan nanofibers have been successfully and verifiably produced. There is one conference abstract describes an attempt to electrospin pullulan and gelatin, however this is the first time it has been chemically confirmed [287].

After choosing our scaffold we tested the composition, degradation rate, and release rate of several polymer combinations. We used combinations of 75% pullulan with

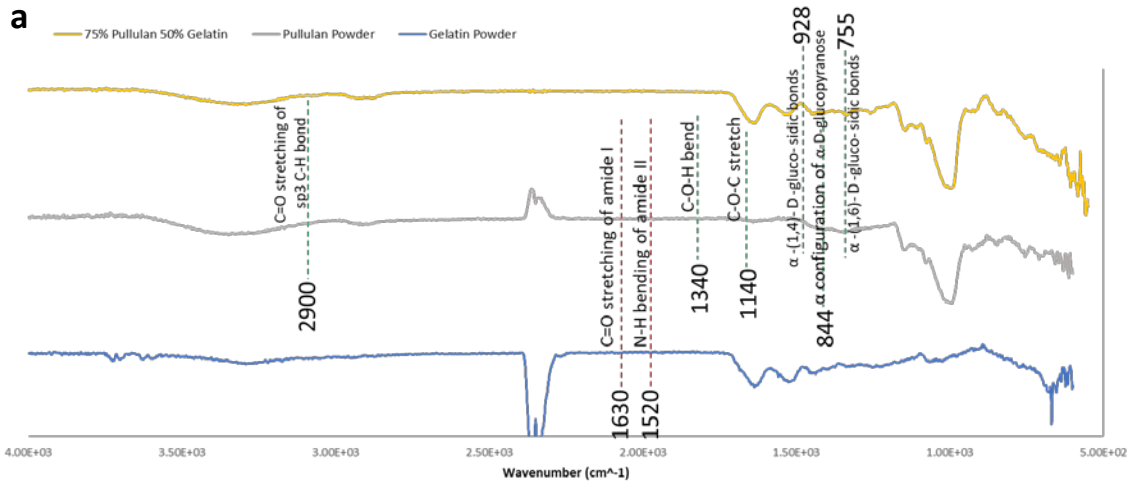
0, 1:70 and 1:50 crosslinking as described in the methods. We also tested 50% pullulan with 0, 1:70 and 1:50. To characterize molecular composition FTIR was performed on all of these including with incorporation of 10µg of RA. As controls, RA, pullulan, and gelatin were each tested, gelatin and pullulan in their powder form, and RA as a KBr pellet. FTIR provides mostly qualitative data showing which materials were present. In each of our samples we confirmed the presence of pullulan, gelatin and loaded drug. In our invitro testing we had to choose one polymer which was 75% pullulan with 1:70 crosslinking, which is why in **Figure 6** we show the FTIR spectrum of only that sample. The 75% pullulan with 1:70 crosslinking sample was decided upon based on degradation and drug release observed. We had also tried using 1:100 crosslinker but it degraded to quickly and could be used invitro.

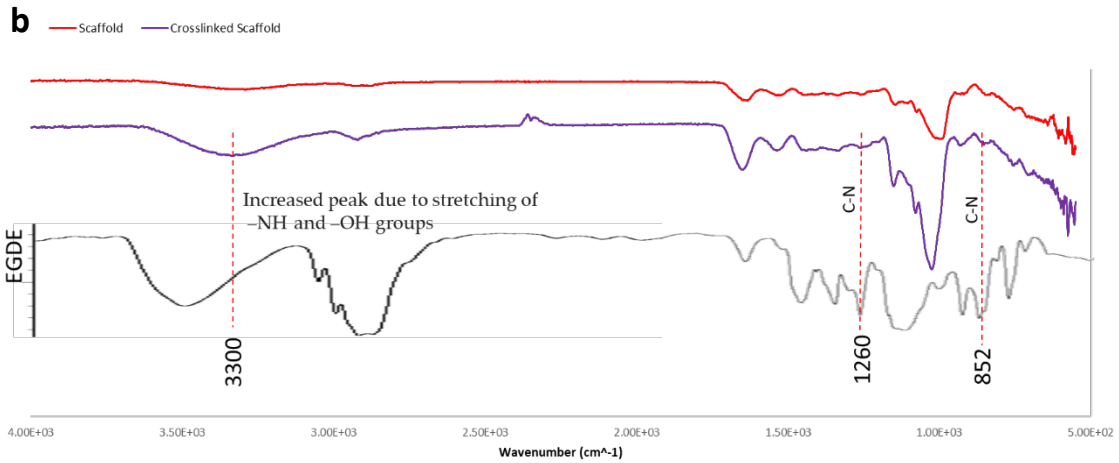
FTIR analysis confirms gelatin and pullulan are both present in this scaffold (Figure 6a). The presence of gelatin can be seen by C = O stretching of amide I at 1630 and N-H bending of amide II at 1520 [288]. The presence of pullulan can be seen by for C=O stretching of sp3 Carbon hydrogen bond at 2900, C – O – H bending at 1340, C – O – C stretching at 1140, α-(1,4)-D-glucosidic bonds at 928, α configuration of α-D-glucopyranose at 844, and α-(1,6)-D-glucosidic bonds at 755 [289], [290]. With the confirmation of the scaffold composition next we tested to see if the crosslinker was still present.

In Figure 6b we confirmed the presence of EGDE in the scaffold. EGDE can chemically react with amino, carboxyl and hydroxyl functional groups. EGDE can be seen by the presence of carbon nitrogen bonds at 852 and 1260 as well as an increased peak

at 330 due to stretching of the hydroxyl group. The IR spectrum for EGDE was taken from the "Spectral Database for Organ Compounds" and superimposed on or graph to determine peak overlap. EGDE is a able to crosslink the hydroxyl groups on both pullulan and gelatin as depicted in figure 3 [150], [151], [218]. EGDE has two reactive epoxide functional groups which are highly reactive due to the strain existing in the epoxide ring.

The detection of RA determined using FTIR to ensure RA was being loaded in the scaffold. RA is a reactive metabolite of vitamin A has several developmental and cellular affects. RA binds to receptors in immune cell and is able to promotes differentiation of myeloid cells into macrophages, reduce inflammation, and convert macrophages in M1 phenotype to M2 phenotype [14], [291]. After crosslinking the scaffold RA was added. The carbon oxygen stretch vibrations at 1250 confirm the presence of RA in Figure 6c [259]





Ethylene Glycol Diglycidyl Ether from the Spectral Database for Organic Compounds
http://sdbs.db.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi

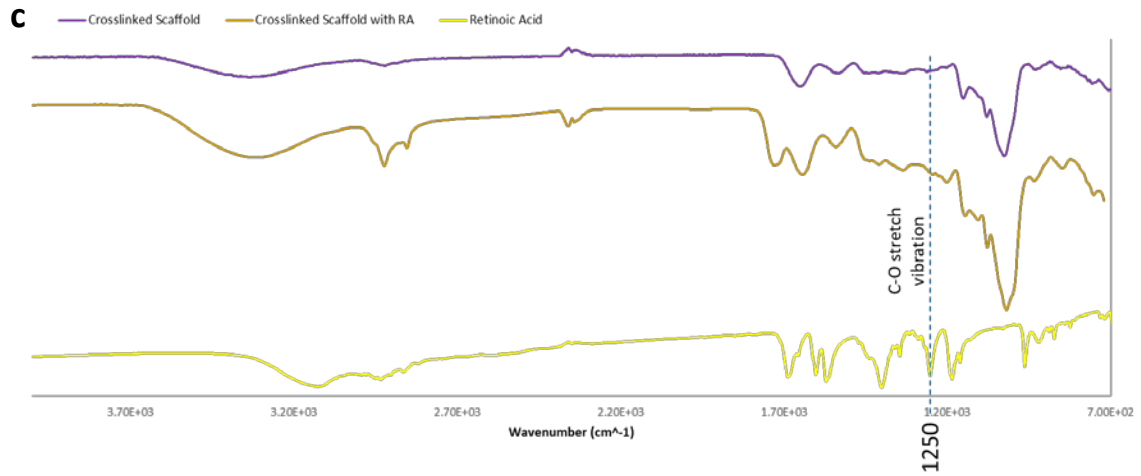


Figure 6: FTIR of pullulan, gelatin, and 75% pullulan/25% gelatin scaffold (a), FTIR of 75% pullulan/25% gelatin scaffold before and after crosslinking 1:70 EGDE in EtOH (b), FTIR of 75% pullulan/25% gelatin scaffold crosslinked 1:70 EGDE in EtOH before and after adding RA (c)

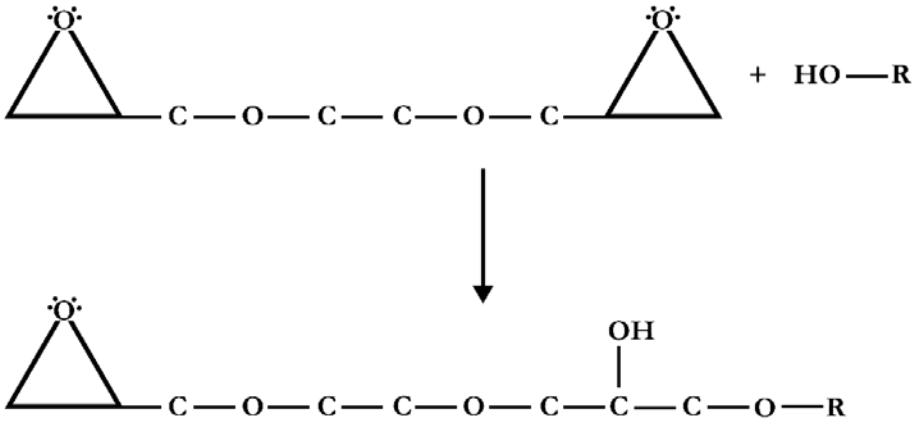


Figure 7: Crosslinking Reaction of EGDE

4.2 Nanofiber Characterization

Each of the polymer nanofiber combinations were imaged using SEM. We looked at 100% pullulan, 75% pullulan and 50% pullulan (75% pullulan means 25% gelatin and so on). The nanofibers were also crosslinked with EGDE 1:70 and 1:50 as in the FTIR. The images shown in **Figure 8** are similar visual between each sample. Nanofibers were seen to be randomly distributed and monomodal. Images were then analyzed using Image J to determine the diameter of the nanofibers as shown in **Table 2**. The diameter of the nanofibers was between 300-370nm for each of the samples. There was a significant increase in diameter as percentage of gelatin increased (**Figure 8 a,b,c**). 75% pullulan with 1:70 crosslinking trends to have the smallest diameter, and smaller diameter size implies

increased space for drug loading, therefore, this presented evidence that it may be a good polymer for drug delivery.

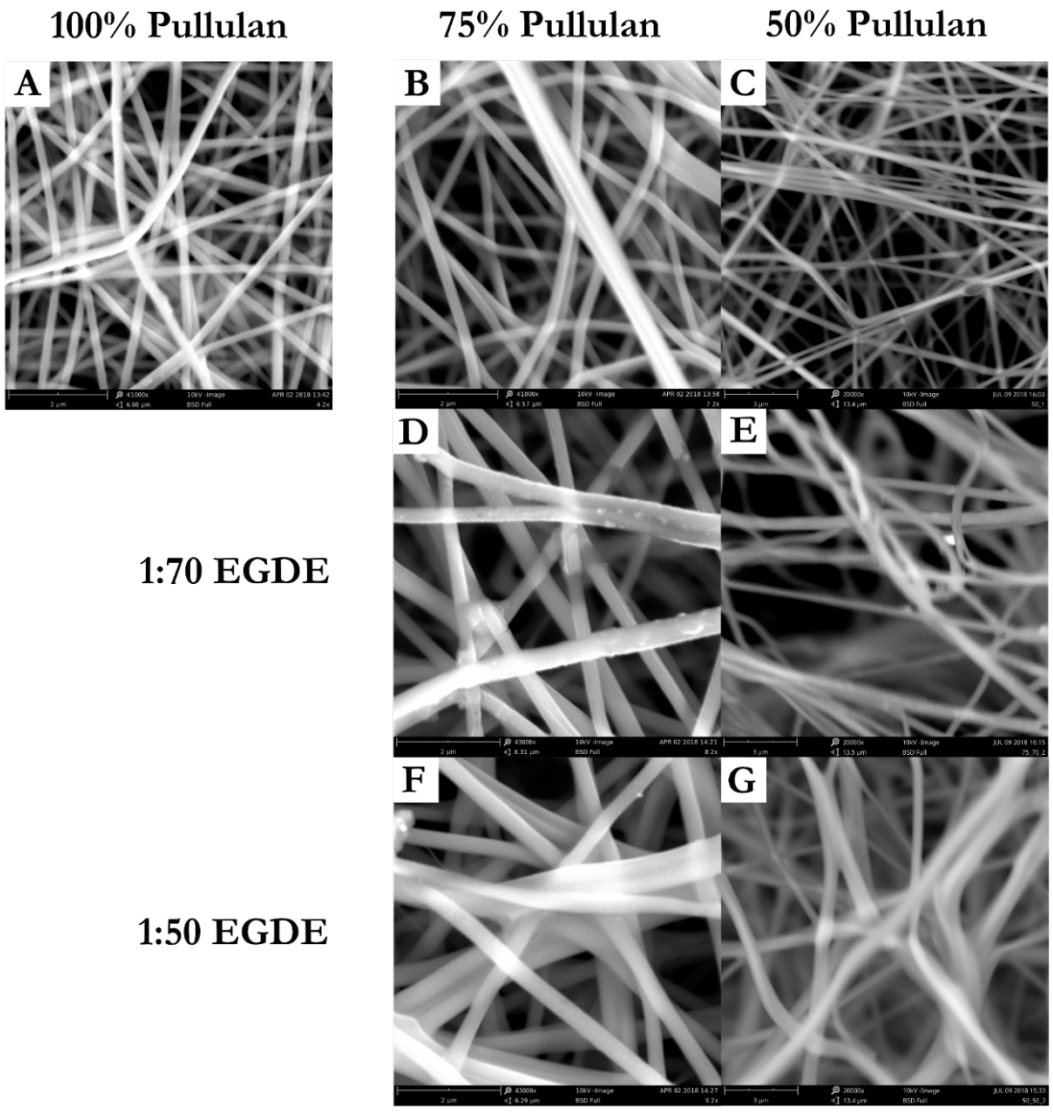


Figure 8: SEM images of nanofibers; 100% pullulan (a), 75% pullulan/25% gelatin (b), 50% pullulan/25% gelatin (c), 75% pullulan/25% gelatin with EGDE crosslinking 1:70 in EtOH 1:70 (d), 50% pullulan/25% gelatin with EGDE crosslinking 1:70 in EtOH (e), 75% pullulan/25% gelatin with EGDE crosslinking 1:50 in EtOH (f), 50% pullulan/25% gelatin with EGDE crosslinking 1:50 in EtOH (g)

Crosslinker Concentration	Pullulan Concentration	Mean Diameter (nm)	St Dev
0	100%	139.4	17.4
	75%	297.5	68.3
	50%	358.3	59.3
1:70 EGDE	75%	328	47.9
	50%	328.5	74.1
1:50 EGDE	75%	352.3	31.3
	50%	362.1	93.7

Table 2: Nanofiber Diameter

4.3 Scaffold Degradation

Degradation testing was performed by incubating samples with Ringers solution, which is an isotonic solution made of sodium chloride, potassium chloride, sodium bicarbonate and calcium chloride. To recapitulate physiological conditions by incubating samples in Ringer's solution and incubating at 37^{o}c with constant agitation. Ringers solution is relatively like body fluids. RA was also added to the scaffolds and examined to see if there was any noticeable effect, however there was no significant effect seen on any of the samples. It was observed that most of the sample degraded after the first day, however it slowed down considerably in the following days and weeks. The reason

degradation was so high at the beginning and then slowed down considerably may have been due to decrease in surface area of the polymer. As the polymer degraded the highly porous scaffold may have shrunk reducing hydrolytic degradation of the polymers [292], [293]. When we examined the degradation via ANOVA 75% pullulan compositions had significantly less degradation compared to 50% pullulan compositions with a p value of <0.0005 that they were different. We decided to go with the polymer that degraded the least so that it would have a higher potential for prolonged drug delivery, therefore, we chose to use 75% pullulan for our invitro studies.

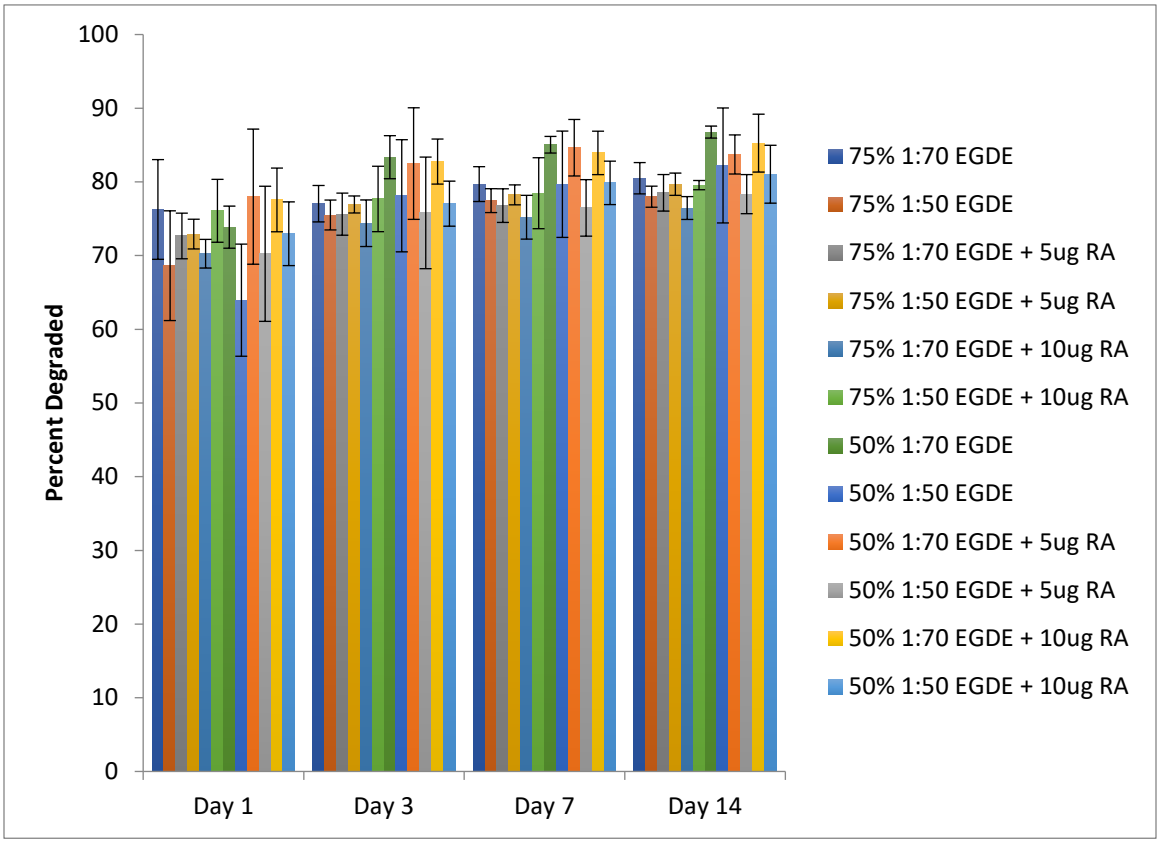


Figure 9: Degradation of scaffold measured on day 1, 3, 7 and 14

4.4 Drug Release

RA release rate was examined at 1h, 2h, 4h, 8h, 1 day, 3 days and 1 week by collecting Ringer's solution from the degradation test samples and testing absorbance at 316nm. Samples were either loaded with 5µg or 10µg of RA. Two concentrations of RA were loaded in the scaffolds 5µg and 10µg. The majority of RA was released within the first 24 hours. Comparing the samples via Two-Way ANOVA, RA is seen to have less release of 1:70 crosslinking than 1:50 crosslinking with a p value of <0.0001. 75% and 50% RA show similar release rates, so composition of pullulan to gelatin does not seem to affect release rate. Since RA has a lower release rate the 1:70 crosslinking we decided to go with this with the idea that drug treatment may be extended.

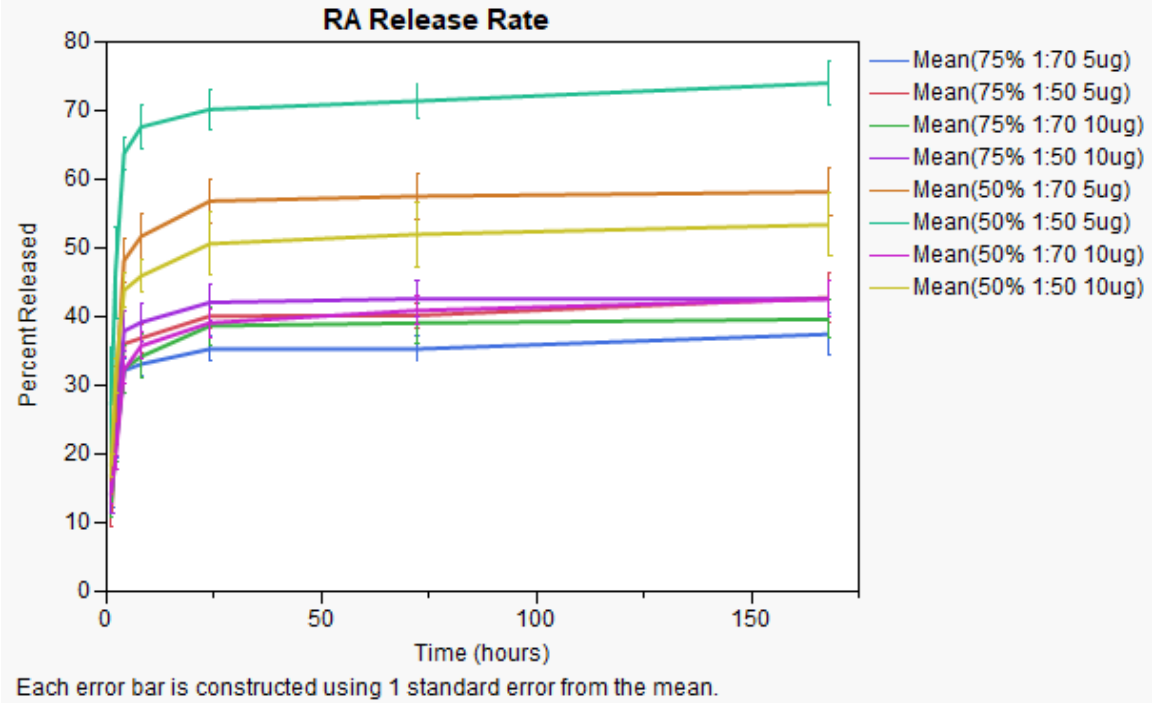


Figure 10: Release rate of RA from scaffold measured at 1h, 4h, 8h, 72h, 168h

4.5 M1 Macrophage Viability and Expression

The inflammatory M1 phenotype causes cell death and delayed healing. Therefore, converting M1 macrophages into M2 may be able to ameliorate inflammatory disorders in which the M1 phenotype is overexpressed such as in chronic wounds. To assess treatment of RA and MSC loaded nanofibrous scaffold to convert M1 macrophages from M1 to M2 phenotype, we performed qPCR and determined M1 and M2 inflammatory marker expression. We chose to use THP-1 cells to derive our macrophages for our invitro studies. THP-1 cells are commonly used to study macrophage functions. We used a

tradition method of using PMA to differentiate the promonocytic THP-1 cells into macrophages as has been well discussed in literature [4]. ThP-1 derived macrophages were then incubated with LPS and IFN-γ to induce M1 phenotype. Prior to doing qPCR with the treatments we first tested the length of time needed to incubate LPS and IFN-γ before M1 markers were expressed and to determine how the cells did with the treatment using a live/dead ethidium/calcein kit. The Viability test (Figure 11) showed a large increase in cells incubated for 2 or three days. qPCR however did not show any differences between 1, 2 or 3 days of treatment with LPS/IFNY (Figure 12 a-c). Important to note is that the M2 marker CCL18 was overly expressed (data not shown). We were using a large amount of LPS to stimulate M1 macrophage phenotype which can cause this to occur [4]. Since there was less cell death at day 1 we chose this for M1 polarization. Next treatment efficacy was assessed.

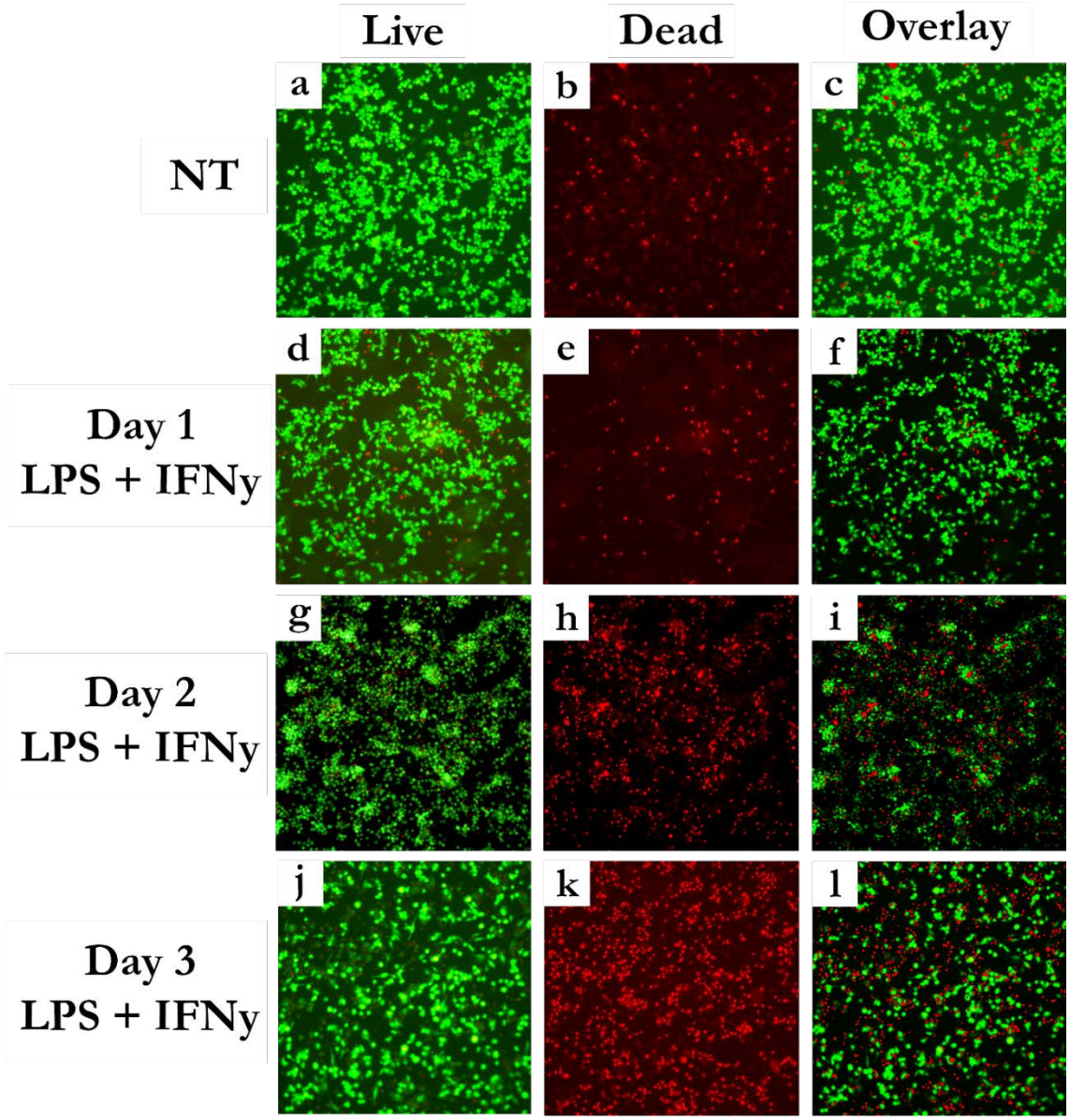


Figure 11: Live/dead assay showing NT (a-c), 1 day of LPS/IFN-γ treatment (d-f), 2 day of LPS/IFN-γ treatment (g-i), 3 day of LPS/IFN-γ treatment (j-l). live cells are depicted in green (a, d, g, j), dead cells are depicted in red (b, e, h, k), and merge (c, f, I, l)

4.6 Retinoic Acid and MSCs Promote M1 to M2 Conversion

To investigate the ability for the RA and ADMSCs to re-polarize macrophages we then conducted invitro tests by incubating the macrophages with scaffolds, then assessed M1 and M2 expression patterns. IL1β and TNFα were the M1 makers tested, and CCl22 is the M2 marker examined. Macrophages are plastic cells and can switch polarity between M1 and M2 phenotype due to environmental factors. Both RA and MSC's have been shown to modulate the response in previous research. In our study RA significantly decreases M1 polarity marker IL-1β, with a p value of 0.0197 (**Figure 12 d-f**). The pullulan/gelatin scaffold was able to significantly decrease all M1 markers and significantly increase all M2 markers. 10µg RA loaded scaffold and MSC loaded scaffold both have increased CCL22, while all treatments can significantly decreased IL1-β, and TNF-α compared to NT (Figure 8 g-i). All treatments were able to decrease inflammatory markers TNFα and IL-1β. Treatment with both mesenchymal stem cells and retinoic seemed the least effective to promote inflammatory cytokine CCL22 and there was no significant difference from the NT. Scaffold, scaffold with MSCs and scaffold loaded with RA were however able to significantly improve CCL22 expression. Perhaps RA is interacting in a way with MSCs that causes a reduction in CCL22 expression. This study reveals modulation of macrophages to M2 phenotype which could promote alleviation of several diseases. Future studies should examine the effect of these different combinations *in vivo*.

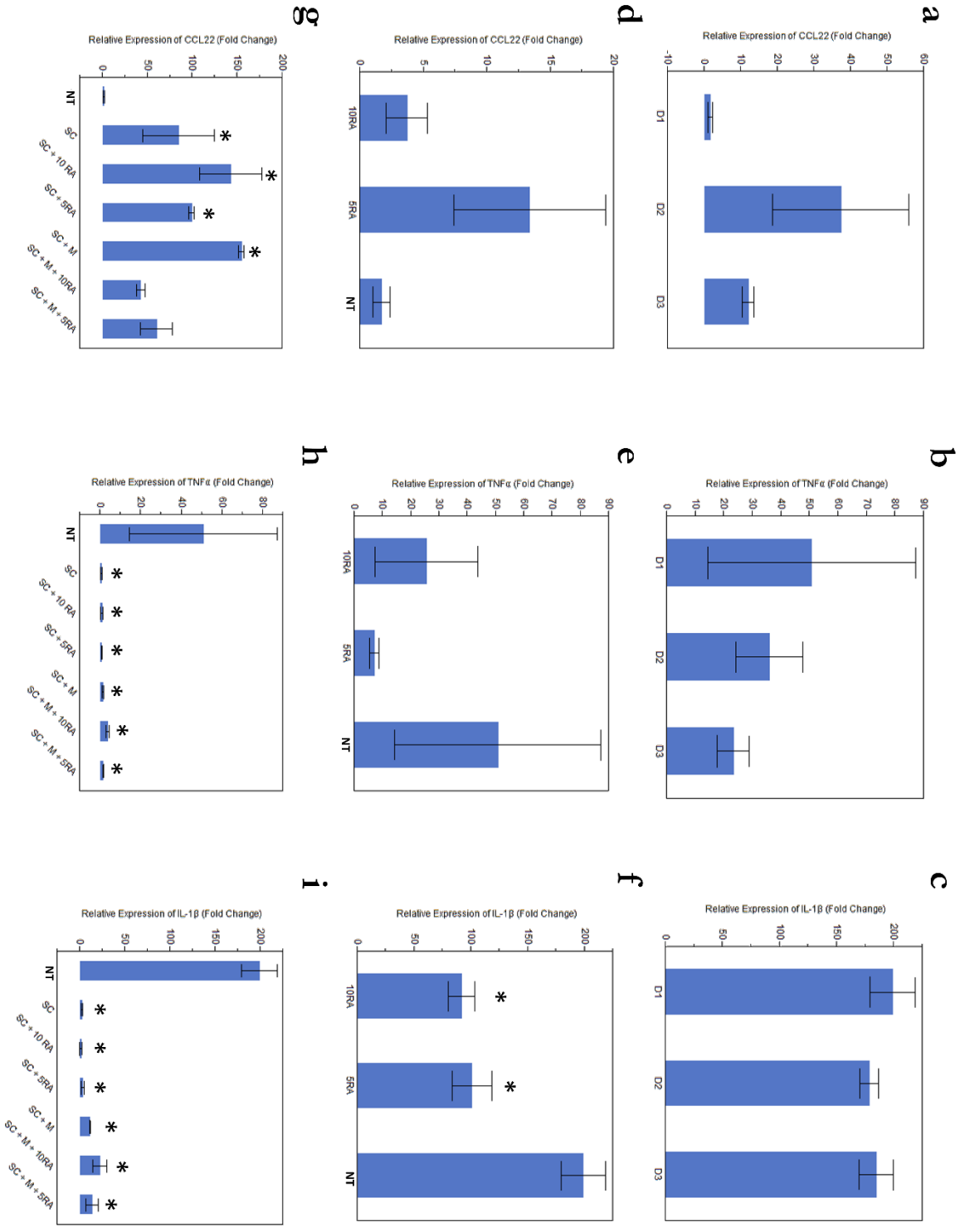


Figure 12: qPCR of 1 day, 2 days and 3 days treatment of macrophages with 100ng M1 to M2 transitioning effect of 100ηg/mL LPS and 100 ηg/mL IFN-γ (a-c), qPCR of RA in vitro on M1 to M2 transition (d-f), qPCR of scaffolds loaded with RA and/or MSCs on M1 to M2 transition (g-i)

4.7 CONCLUSIONS

In summary, electrospun scaffolds composed of pullulan and gelatin nanofibers were successfully fabricated and crosslinked with EGDE, and loaded with RA. This work demonstrates the ability of RA, MSCs and the scaffold by itself to induce a shift from a M1 into an M2 phenotype in activated macrophages. Inflammatory markers IL1β and TNFα were decreased in all samples. All cells treated with RA and the scaffold containing MSCs were able to increase the M2 marker CCL22. MSCs and RA treated wells however did not have significantly improved CCL22. Further examination should be done to determine the reason why there was a CCL22 decrease in cells treated with both RA and MSCs. These tests verify the feasibility of combining this 75% pullulan and 25% gelatin electrospun scaffold with RA and MSCs to convert M1 macrophages into M2 phenotype, thus having potentially as novel therapeutic to reduce inflammatory diseases such as chronic wounds.

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