A SYNERGISTIC APPROACH TO DEVELOP ELECTROSPUN WOUND DRESSINGS USING HONEY AND *S*-NITROSO-*N*-ACETYLPENICILLAMINE

by

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(Under the Direction of Hitesh Handa)

ABSTRACT

The healing of a skin wound is a complex process requiring the joint efforts of many different tissues and cell lineages, and a major concern of the healing process is bacterial infection. Wounds lead to exposure of subcutaneous tissue which provides a nutritious environment conducive to microbial colonization and proliferation. The presence of bacteria at the wound site down-regulates the host's immune response and ultimately delays the natural wound healing process. This leads to a great demand for wound dressings that have antibacterial properties, and positively affect the inflammation, proliferation and remodeling stages of the healing process, while also providing a protective barrier.

In this study, we developed a biocompatible and antibacterial electrospun wound dressing. A double healing factor system was designed by using the electrospinning method, where the ancient healing medicine – honey was loaded with the Nitric Oxide (NO) donor, – *S*-Nitroso-*N*-acetylpenicillamine (SNAP) to a Polylactic acid (PLA) fiber. Honey is one of nature’s wound healing agents due to its complex composition and high sugar level, it has antibacterial potential, immunomodulatory effect, antioxidant capacity

and high biocompatibility. The fact that honey can accelerate wound repairing has been proven in countless studies. Furthermore, in the last two decades, NO has emerged as a critical player in all four phases of natural wound healing namely hemostasis, inflammation, fibroblast proliferation, and tissue remodeling. Because of its rapid action, short-half-life, and nonspecific action it does not promote antibacterial resistance.

The synergistic effects of honey and NO donor, SNAP was studied by fabricating four types of fibers: PLA, PLA-Honey, PLA-SNAP and PLA-Honey-SNAP. The combination of honey and SNAP demonstrated the best antibacterial ability without being cytotoxic. Also, with the presence of honey, the fiber showed strong antioxidant ability, which is a beneficial physical property, and can speed-up the wound healing.

INDEX WORDS: Nitric Oxide, Antimicrobial, Wound healing, Electrospun Nanofiber

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CHAPTER 1

INTRODUCTION

1.1 Wound Healing

The healing of a skin wound is a complex process requiring the joint efforts of many different tissues and cell lineages. The scientific definition of "wound" is the disruption of typical anatomic structure and function, and it is a result of pathologic processes beginning internally or externally to the involved organ [1]. For several decades, several research groups have been working on strategies to promote the process of wound healing. Wound healing has four phases: (i) coagulation and hemostasis; (ii) inflammation; (iii) proliferation; and (iv) wound remodeling with scar tissue formation [2]. It involves the interactions of many different types of cells and matrix components to establish a provisional tissue and eventually an entire regenerated epidermis [3].

The first stage is clot formation, which can stop the bleeding of blood from wounded vessels. A clot consists of platelets, which are embedded in a cross-linked fibrin fibrous network produced by thrombin cleavage of fibrinogen, and a small amount of plasma fibronectin, vitronectin, and thrombospondin [4]. It can become a temporary shield to protect the wounded tissue and provides a provisional matrix for cells migrating during the repair process.

The next stage is inflammation. In the beginning, a variety of chemotactic signals are released from the wound sites; those signals are produced not only by the degranulating platelets, but also from cleaved bacterial proteins, by-products of

proteolysis of fibrin and other matrix components [4]. In response to the molecular changes, both neutrophils and monocytes can be recruited from the circulating blood and attracted to the surface of endothelial cells lining capillaries. Neutrophils arrive fast and clear the initial rush of contaminating microorganisms, and macrophages can consume all the remaining pathogenic organisms and other cell and matrix debris. Both neutrophils and macrophages can release a series of growth factors and cytokines at the wound site to amplify the signals. In routine skin wound healing, the inflammation usually lasts 2 to 5 days, and once the harmful irritation is eliminated, the inflammation stops [5].

Proliferation happens after the inflammation subsides; this is a process to cover the wound surface, restore the vascular network, and form granulation tissue. Fibroblasts proliferate and produce extracellular matrix (ECM) proteins, which can further support cell migration as it releases growth factors and provides support for cell adhesion. Collagens are also synthesized by fibroblasts and impart integrity and strength to tissues [2].

Re-epithelialization requires migration and proliferation of keratinocytes. The migrating cells are released from their original sites by collagenase and elastase, triggered by a loss of contact inhibition and physical tension at cell adhesion structures. This process stops when the cells get in contact, and new adhesion structures are formed, and keratinocytes secrete proteins to rebuild the basement membrane [6]. The restoration of the blood vessels network is initiated by different growth factors, and this process is also known as "angiogenesis", which is very important since nutrients and oxygen are needed during wound repair [7]. The growth factors can activate the endothelial cells of existing vessels and can escape from the existing vessels, then proliferate and migrate towards the

source of the angiogenic stimulus. These sprouts can form vessel lumen, differentiate into arteries and venules, and mature by recruitment of pericytes and smooth muscle cells [8]. In this phase, the provisional wound matrix formed during haemostasis is replaced by granulation tissue, which is composed of macrophages, fibroblasts, vascularization, collagen matrix, fibrinogen, fibronectin, hyaluronic acid [9].

The remodeling phase starts at the end of the granulation tissue development, and this can last up to 1 or 2 years. Fibroblasts differentiate into myofibroblasts driven by mechanical tension and cytokines, which can contract the wound [10] and undergo apoptosis when healing is complete [11]. The initial deposition of collagen bundles is highly disorganized, it becomes more oriented and cross-linked over time, while the growth of capillaries stops, blood flow declines, and metabolic activity at the wound site decreases. The result is a fully mature scar with a high tensile strength on the skin.

Wound healing is a complex process with many potential factors that may delay healing, and there has been increasing concern toward bacterial effects over the past few years.

1.2 Infections and Biofilms

Skin is the shield for external pathogens, and wounds can cause it to lose its inherent barrier functions, that's why bacterial infections frequently occur at the wound sites. The wounded environment can facilitate the development of microbial communities, the biofilms, which are associated with both wound infections and those wounds that fail to heal. Both acute and chronic wounds can get infected by biofilms, but mostly occur chronic wounds [12, 13]. If most non-healing wounds contain biofilms, this

could be the cause of billions of dollars in global costs for chronic wounds [14]. Local wound infection and foreign bodies can prolong the inflammatory phase, which can interfere with epithelialization, contraction, and collagen deposition. The bacterial endotoxins themselves trigger phagocytosis and the release of collagenase, which can cause collagen degradation and destroy previously normal tissues around it [15]. And the wound contamination associated with tissue hypoxia may inhibit macrophage-regulated fibroblast proliferation [16].

General Bacterial infection happens quite often in our daily life activities, and it can cause a series of health problems. The infectious bacteria can multiply quickly in the human body, and release toxins that can damage cells and tissues. Some common infectious organisms include Gram-negative Escherichia coli (*E. coli*), Pseudomonas aeruginosa (*P. aeruginosa*), and Proteus mirabilis, and the infamous Gram-positive bacteria, methicillin-resistant Staphylococcus aureus (MRSA) [17]. Although those bacteria exist in our daily environment, like in water, on food, on our skin or on surfaces and equipment that are not adequately cleaned, it can be severe once the host's immune system is weakened or has an open skin wound. When microbial onslaught overwhelms the host defense, infection ensues. In the USA, over 17 million people develop a chronic infection annually, and 500, 000 people die with or from a chronic infection each year [18].

In recent years, bacterial biofilms have gained increasing attention as microorganisms are ubiquitous in a variety of environments. The matrix formed in biofilms can protect bacteria from desiccation, biocides, antibiotics, heavy metals, ultraviolet radiation, the host’s immune defense, and many protozoan grazers [12]. So, it makes the infection more difficult to mitigate and cure.

Biofilms can form on the surface of wound sites or medical devices after bacterial colonization, and aggregate into a hydrated polymeric matrix. The secreted matrix can support a stronger adhesion to the surface and provide a protective barrier from antimicrobial penetration and macrophage engulfment [19]. Once these sessile communities and their inherent resistance are formed, it can lead to persistent and chronic infections [20]. The dynamic life cycle of biofilm on a medical device can be divided into five phases: (1) transport and initial attachment of microbes, (2) irreversible adhesion or attachment, (3) microcolony formation, (4) maturation of the biofilm, and (5) detachment and dispersion of the cells [21]. After the biofilm is formed, its maturation is dependent on signaling molecules between cells and a phenomenon known as quorum sensing. Though this way, bacteria can work as a single unit and react to the surrounding environment [22]. As the bacteria continuously grow and colonize, some cells may detach and disperse into the biological environment leading to the spread of infection within the host.

A significant number of researches have indicated that bacterial biofilms are present in the setting of several chronic wound sites, including pressure sores, diabetic foot ulcers, and venous stasis ulcers. One group has demonstrated that biofilm can significantly delay re-epithelialization in a murine cutaneous wound model [23]. This represents direct evidence for the effect of biofilms on wound healing.

In a clinical setting, basic wound cleansing is an important physical method to remove organisms and their byproducts from the wound site. Debridement can not only directly reduce the bacterial bioburden and their secreted toxins, but also remove debris and deactivated tissues, thereby reducing the nutritional source of residual bacteria [24].

Other than that, we need more convenient and advanced methods to prevent bacterial infection and assist in wound healing.

1.3 Antibiotics and Resistant Bacteria

For the medical treatment of bacterial infections, the clinical standard is the use of antibiotics. The first antibiotic, penicillin, was discovered in 1928, then the golden age of antibiotics started in the early 1940's [25]. Yet, due to the widespread use of antibiotics for many years, bacterial resistance has prevailed, and resistant strains have continued to increase.

One example of resistant bacteria is *S. aureus*, which is a leading cause of nosocomial infections, including bacteremia, surgical wound infections, as well as pneumonia [26-28]. About 20% of healthy people always carry one or more strains asymptomatically [29]. One of the best therapeutic choices for the treatment of *S. aureus* is by the administration of methicillin, but it has shown multi-drug resistance for nearly 50 years [30]. The numbers of MRSA strains have significantly increased [31-33]. In 2002, 57% of *S. aureus* isolated was presented as MRSA [34]. The spread of MRSA prompted vancomycin as the first-line drug for the treatment of *S. aureus* infections, but resistance has been quickly established, leading to vancomycin-resistant *S. aureus* (VRSA) [35]. Although VRSA is rarely compared to MRSA, the alarm has been raised in the medical community as *S. aureus* causes life-threatening infections in hospitalized and non-hospitalized patients [36]. Moreover, bacteria residing within biofilms have shown over 1,000 times more resistance to antibiotics [23].

Many facilities now require the identification of infected organisms before deciding on medical care. From a pharmaceutical perspective, the investment in antibiotics has been reduced because of the high risk and estimated inevitability of resistance within 2 years of use [13]. Therefore, new avenues for treatment and prevention of bacterial infections are continually being pursued by scientists.

1.4 Strategies for Wound Dressings

To overcome the problems of wound infections, scientists have developed a series of biomedical materials for wound healing. These materials have the function of wound dressing, which can prevent bacterial invasion, assist and accelerate the healing process, and can be loaded with specific drugs as a delivery system. The characteristics of an ideal wound dressing should be: a) provide or maintain a moist environment; b) enhance epidermal migration; c) promote angiogenesis and connective tissue synthesis; d) allow gas exchange between wounded tissue and the environment; e) maintain appropriate tissue temperature to improve the blood flow to the wound bed and enhance epidermal migration; f) provide protection against bacterial infection; g) should be non-adherent to the wound and easy to remove after healing; h) must provide debridement action to enhance leucocytes migration and support the accumulation of enzymes; i) must be sterile, non-toxic and non-allergenic [37].

The development of wound healing material has a long history. From ancient times, wet-to-dry dressings have been used widely for wound debridement. The earliest prototype of bandages can be traced back to 1600 BC, linen strips soaked in oil or grease covered with plasters was used to occlude wounds. Since then, there have been increasing

records of the use of different materials to assist with wound healing, such as clay tablets, honey or resin and wool boiled with wine [37].

During the 19th century after penicillin was discovered, the major breakthrough, antibiotics, were also introduced to the field of wound healing. Modern wound dressing started development in the 20th century. Nowadays, there are more than 5,000 wound care products in the market [38]. The modern wound dressings are designed to not only cover the wound, but also facilitate its function. Based on the cause and type of wound, numerous materials are available with different functional focus, but the general goal is keeping the wound from dehydration and to promote healing. There are passive products like gauze and tulle, and interactive dressings which are semi-occlusive or occlusive such as films, foam, hydrogel and hydrocolloids. These dressings act as a shield against penetration of bacteria to the wound environment.

Moisture retentive dressing materials can reduce the incidence of wound infection, by maintaining a moist environment, it can help optimize neutrophil activity and reduce the amount of dry necrotic tissue and dead space that facilitates bacterial growth [39]. The use of topical antimicrobial agents can further reduce bioburden and improve wound healing response. However, antibacterial agents alone cannot affect existing bacterial toxins, and bacterial death may lead to increased endotoxin release [40]. In addition to antibacterial effects, the use of topical antibacterial agents (including silver, iodine and chlorhexidine) may also have a beneficial effect on healing. For example, for silver sulfadiazine, it has been shown to accelerate the healing process, and zinc may be the active wound healing component in bacitracin zinc [16, 41]. Aside from traditional antibiotics, other measures are implemented to prevent and treat the wound infections.

Based on recent studies, Nanoparticles (NPs) are considered a promising alternative to traditional antibiotics, since they display antibacterial activity on a large number of strains and can minimize the adverse side effects of the drug, without triggering microbial resistance [42]. Due to the intrinsic properties, NPs have been used in different therapeutic approaches including wound healing.

NPs can direct contact with the bacterial cell wall then release toxic metal ions or generation of Reactive Oxygen Species (ROS) to perform their bactericidal effect [43]. The positively charged NPs are attracted to a bacteria's surface by the negatively charged groups (lipopolysaccharides in gram-negative and teichoic acid/peptidoglycan in gram-positive), then they can establish van der Waals forces, receptor-ligand, and hydrophobic interactions with the cell wall. Under the alteration of permeability, the cell wall can be disrupted and consequently lose the intracellular components [44].

Silver nanoparticles (AgNP) have gained widespread attention among the available NPs, because of their broad inhibitory activity against nearly 650 microorganisms, and also antibiotic-resistant bacteria [45]. The application of silver nanoparticles as a healing factor in wound dressings has been reported in a great number of literatures. It has exhibited strong antimicrobial activity against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) by being used along with [46] or loaded in other materials, such as hydrogel [47], nano-fibrous [48, 49] and bacterial cellulose [50]. With different carriers, these materials have demonstrated the advantages of additional physical characterizations, such as better mechanical property and water absorption ability. But the biggest concern of NPs is its cytotoxicity, due to the small size, it can easily enter the human body and cross biological barriers. It may reach and disrupt the cells normal

biochemical pathways within the most sensitive organs [51, 52]. So, in recent studies the use of metal nanocomposites has been combined with natural products to enhance their biocompatibility [53].

Beside the nanoparticles, more and more natural sources have been used to increase the antimicrobial activity and the biocompatibility in wound healing materials. Honey, essential oils, alginate and chitosan are representative of some natural antibacterial agents [54]. Among all those agents, honey has been incorporated into wound dressings since ancient times. It has shown a series of great properties for healing processes, antibacterial activity, debriding activity, minimize inflammation and stimulate angiogenesis, granulation, wound contraction and epithelialization, and capacity to perform topical nutrition to the wound [55, 56].

The antimicrobial activity of honey is attributed to its acidity, low water content, high sugar level, and presence of antimicrobial substances such as hydrogen peroxide, antimicrobial peptide bee defensin-1, flavonoids, and phenolic acids [57, 58]. The gluconic acid in honey provides an acidic PH, which may aid macrophages to kill bacteria and prevent biofilm formation [59]. Furthermore, the low water content and high osmolarity can create a difficult environment for bacterial survival and growth [60]. Hydrogen peroxide, which is one of the components of honey, can react with the cell wall, lipids, proteins and nucleic acids available in bacteria to inhibit its growth [61]. Honey's antibacterial activity against *E. coli* and *S. aureus* have been shown in several studies as an agent in wound dressings [62, 63]. And honey also shows an anti-inflammatory and antioxidant capability, which can also accelerate the healing process [60].

1.5 Background on Nitric Oxide

Nitric oxide (NO) has been widely used over decades since it was identified by Furchgott, Ignarro, and Murad in 1987 [64]. It was first described as a potent vasodilator secreted by the healthy endothelium that can inhibit platelet adhesion and aggregation to the blood vessel wall [65]. In 1992, the free radical NO received approbation as "molecule of the year" by the journal Science and was the subject of the Nobel Prize in Medicine and Physiology. Numerous reviews and studies have been published devoted to different NO releasing / generating materials and their many potential biomedical applications.

As a gaseous free radical molecule, NO has been identified as a potent antimicrobial agent, a mediator of smooth muscle relaxation and platelet inhibitor. It is self-produced in mammalian cells by nitric oxide synthase (NOS), with the conversion of L-arginine to L-citrulline enzymatically in the presence of molecular oxygen [66]. There are three isoforms of NOS in various areas of the body, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), they all are essential factors in the cardiovascular system [67]. The differences between these enzymes are their utilization of calcium, eNOS and nNOS are calcium-dependent, they can generate low and continuous levels of NO release; while iNOS is calcium-independent, it can generate high levels of NO when needed [68]. Due to the small size and nearly zero charge, NO molecule can diffuse across membranes easily. Once it enters the bacteria, NO can form reactive oxygen species intermediates in situ or directly target DNA and microbial proteins. In an indirect pathway, NO can react with superoxide to generate a potent oxidizer intermediate, peroxynitrite ($OONO^-$), which can also cross the bacterial membrane and target cellular components leading to enzyme inhibition, lipid peroxidation and protein

nitrosation [69]. In addition to planktonic bacteria, NO has also been shown to be effective against biofilm bacteria. And due to its rapid action, short-half-life, and a broad spectrum of antimicrobial effects NO significantly reduced the probability of microbial resistance [70].

Evidence has shown that all three NOS enzymes are found to be associated with the wound healing process, which means NO has played a critical role in natural wound healing [68]. Further studies have proven the effect of NO in promoting the healing process, including angiogenesis, vasodilation, cell proliferation, inflammation, collagen synthesis, and tissue remodeling [68, 71, 72].

The enticing antimicrobial and wound healing traits of NO make it valued by scientists, and it has been used to design a variety of biomedical materials. It is a challenge to delivery NO to the body caused by its short half-life in vivo, and this issue has led to the development of donor molecules. Many compounds with nitrogen-oxygen bonds can decompose and generate reactive nitrogen species under the presence of light, moisture, or heat [73]. And some donors are pH-dependent or require catalyst addition Recently, several widely used NO donors are S-nitrosothiols, diazeniumdiolates, organic nitrates/nitrites, and metal-NO complexes.

Organic nitrates and nitrites have been used in the cardiovascular field for treating blood vessels and heart disease for a long time, but they are limited by their short half-life and drug resistance [74]. Diazeniumdiolates (NONOates) was developed to overcome these limitations, its structure allows for two moles of NO to be attached, which is highly efficient, but it is still unstable in physiological pH and temperature for long term release. S-nitrosothiols (RSNOs) naturally occurs and can be synthetized, it can cleave it's NO

group in the forms of NO•, NO−, and NO+ in thermal conditions, or with a metal ion catalyst [68]. A synthetic RSNO applied broadly in preclinical studies is called *S*-nitroso-*N*-acetylpenicillamine (SNAP), which has shown an excellent stability and potential for commercial use. SNAP has been proven to have significant antimicrobial activity in both in vitro and in vivo studies [75, 76].

Although no work has shown encouraging results, current manufacturing methods have some disadvantages, which include high production costs, storage stability, and biocompatibility. And for the process of wound healing, an individual factor cannot adequately meet the requirements for every stage, a more versatile, efficient and cheap wound dressing is our goal.

This thesis aims to address the problem of acute wound healing and infection, and the solution we developed, a biocompatible and antibacterial electrospun wound dressing. A novel double healing factor system was designed by using the electrospinning method, where the ancient healing medicine – honey was loaded with the NO donor SNAP to a Polylactic acid (PLA) fiber. Honey provides its immunomodulatory effect, antioxidant capacity and high biocompatibility, while SNAP brings efficient biofilm removal and vascular regeneration promoting ability. The mutual promotion and complementation of the two factors achieved a more optimized healing and sterilization effect. At the same time, the electrospun fiber is not only a drug release system, but also has excellent breathability, water absorption and highly specific surface area which provides a suitable wound healing environment.

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CHAPTER 2

A SYNERGISTIC APPROACH TO DEVELOP ELECTROSPUN WOUND DRESSINGS USING HONEY AND *S*-NITROSO-*N*-ACETYLPENICILLAMINE [1]

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ABSTRACT

The healing of a skin wound is a complex process requiring the joint efforts of many different tissues and cell lineages, and a major concern of the healing process is bacterial infection. Wounds lead to exposure of subcutaneous tissue which provides a nutritious environment conducive to microbial colonization and proliferation. The presence of bacteria at the wound site down-regulates the host's immune response and ultimately delays the natural wound healing process. This leads to a great demand for wound dressings that have antibacterial properties and positively affect the inflammation, proliferation and remodeling stages of the healing process, while also providing a protective barrier.

In this study, we developed a biocompatible and antibacterial electrospun wound dressing. A double healing factor system was designed by using the electrospinning method, where the ancient healing medicine – honey – was loaded with the nitric oxide (NO) donor, – *S*-Nitroso-*N*-acetylpenicillamine (SNAP) to a polylactic acid (PLA) fiber. Honey is one of nature's wound healing agents due to its complex composition and high sugar level, it has antibacterial potential, immunomodulatory effect, antioxidant capacity and high biocompatibility. The fact that honey can accelerate wound repairing has been proven in countless studies. Furthermore, in the last two decades, NO has emerged as a critical player in all four phases of natural wound healing: hemostasis, inflammation, fibroblast proliferation, and tissue remodeling. Because of its rapid action, short-half-life, and nonspecific action, bacteria have shown little resistance against NO.

The synergistic effects of honey and SNAP were studied by fabricating four types of fibers: PLA, PLA-Honey (PLA/HN), PLA-SNAP (PLA/SN) and PLA-SNAP-Honey (PLA/SN/HN). The combination of honey and SNAP demonstrated the best antibacterial

ability without being cytotoxic. Also, with the presence of honey, the nanofibers showed strong antioxidant ability, which is a beneficial physical property, and can speed-up the wound healing.

2.1 Introduction

Acute wound healing involves a complex series of events including chemotaxis, cell division, neovascularization, new extracellular matrix synthesis, and the formation and remodeling of the scar tissue. These events are regulated by several mediators including platelets, inflammatory cells, cytokines, growth factors, and matrix metalloproteinases and their inhibitors [1]. The behavior of each of the contributing cell types in the proliferation, migration, matrix synthesis, and contraction phases, as well as the growth factor and matrix signals appearance at a wound site, are now generally understood [2].

The main concern of the healing process is bacterial infection. Wounds lead to exposure of subcutaneous tissue which provides a nutritious environment conducive to microbial colonization and proliferation. The presence of bacteria at the wound site down-regulates the host's immune response and ultimately delays the natural wound healing process. Bacterial colonization and infection can also cause life-threatening complications like pressure sores and diabetic foot ulcers, which can lead to amputation of the affected limb [3]. Skin infections contribute to about 200 million visits to physicians costing over $350 million annually in the US [4].

Scientists have been looking for treatments to bacterial infections for decades. However, the ever-growing incidence of antibiotic resistant bacteria (e. g. methicillin - resistant *Staphylococcus aureus* (MRSA)), has rendered these treatments less effective, the problem is getting more serious. The current treatment option for MRSA is vancomycin, which is considered the gold standard for the treatment of resistant pathogens. The widespread use of this antibiotic has now led to an emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA) and vancomycin - resistant

enterococcus (VRE), leading to increased mortality [5, 6]. This precipitates a growing alarm among researchers and healthcare providers worldwide.

Therefore, finding a new strategy for fighting bacteria is particularly urgent. This has also led to a great demand for wound dressings that can positively affect the inflammation, proliferation and remodeling stages of the healing process, while also providing a protective barrier.

Honey is a natural wound healing agent. Due to its complex composition and high sugar level, honey has antibacterial potential, immunomodulatory effect, antioxidant capacity and high biocompatibility. The use of honey for wound dressings has been documented back thousands of years in both ancient China and Egypt [7]. The fact that honey can accelerate wound repair has also been proven in recent studies.

The antibacterial properties of honey are obtained through its special properties and composition: (i) high sugar content, (ii) low moisture content, (iii) gluconic acid, creating an acidic environment and (iv) hydrogen peroxide [8]. In addition, honey has also been shown to have significant antioxidative properties [9], which also plays a key role in regulating the wound healing process. Honey can scavenge free radicals and neutralize them after they have been formed in the wound to reduce the damage that would otherwise have resulted from these radicals [10]. With all these great healing properties, we chose to use honey as one of the factors for our wound dressing material.

In the last two decades, nitric oxide has emerged as a critical player in all four phases of natural wound healing namely hemostasis, inflammation, fibroblast proliferation, and tissue remodeling. Because of its rapid action, short-half-life, and nonspecific action it does not promote antibacterial resistance. Furthermore, NO is more

than just an antibacterial agent due to its proven role in angiogenesis, vasodilation, cell proliferation, inflammation, collagen synthesis, and tissue remodeling [11].

Electrospinning is a technique widely used in electrostatic fiber formation. It was first introduced in the early 1930s to make nanofibers, used as filter materials and textile yarns [12]. Because of the high efficiency and excellent biocompatibility, as well as the simple and cheap synthesis method, electrospinning fiber has been widely studied and applied in the field of biomedical materials over decades.

This method uses high-voltage electric fields to produce polymer fibers with diameters ranging from a few nanometers to a few microns. The polymer solution is placed in a syringe, a high-voltage electric field is applied to the head of the needle and a collector screen at certain distance. Under the action of a continuous driving force on the syringe and the electrostatic field, the polymer solution will be sprayed in a filament shape to the collector, with the rapid evaporation of organic solvents, a fiber material will be formed.

Electrospun fibers have been widely used in wound healing application in the last three decades. Because they provide a good substrate for cells to attach and grow due to their large surface area and ECM-like structure [13]. The porous nature of nanofibers is well-suited for drainage of wound exudate and allows atmospheric oxygen to properly penetrate the wound [14]. Nanofibers can also be loaded with drugs and other therapeutic agents. By loading specific factors like antibacterial drugs to the fibers, they can be made to prevent the invasion of microorganisms to avoid possible infections. According to the reports, there are several methods to incorporate active agents including blending,

core/shell fabricating, encapsulating, post-treatment and attachment [15]. Here we selected the blending method because we wanted our factors to have an even and gradual release.

Polylactic acid has been reported to be a biodegradable and biocompatible aliphatic polyester with relatively high strength [16]. Vast research of PLA fibers has shown great results in biomedical applications, including surgical sutures, tissue regeneration scaffolds, and drug delivery carriers.

Here we loaded honey and SNAP in PLA nanofibers to manufacture a double healing factor functioning system for wound dressing application. The novel combination of those two agents showed great antibacterial ability and a low cytotoxicity level, which indicates that we have successfully developed this innovative, effective and versatile biomedical material.

2.2 Materials & Methods

Materials

N-Acetyl-d-penicillamine (NAP), tetrahydrofuran (THF), sodium nitrite (NaNO2), Dimethylformamide, (DMF), ethylenediaminetetraacetic acid (EDTA), hydrochloric acid, and Polylactic Acid (PLA) were purchased from Sigma-Aldrich (St. Louis, MO 63103). Honey was purchased from local Stores (Athens, GA). Phosphate-buffered saline (PBS), pH 7.4, used for *in vitro* experiments contained 138 mM NaCl, 2.7 mM KCl, and 10 mM sodium phosphate. Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA were purchased from Corning (Manassas, VA 20109). The Cell Counting Kit-8 (CCK-8) was purchased from Sigma-Aldrich (St. Louis, MO 63103). Penicillin-Streptomycin (Pen-Strep) and fetal bovine serum (FBS) were obtained from Gibco-Life Technologies (Grand Island, NY 14072). The bacterial strains *S. aureus* (ATCC 6538) and 3T3 Mouse fibroblast cell line (ATCC 1658) was purchased from American Type Culture Collection (ATCC). LB broth was obtained from Fisher Bioreagents (Fair Lawn, NJ). LB Agar was purchased from Difco Laboratories Inc. (Detroit, MI). The lactate dehydrogenase (LDH) kit was purchased from Roche Life Sciences (Indianapolis, IN). Glutathione reduced was purchased from Goldbio (St. Louis, MO).

SNAP Synthesis

SNAP was synthesized according to a previously established protocol with slight modifications [17]. Briefly, an equimolar ratio of NAP and NaNO2 were added to a beaker containing a 1:1 mixture of DI water and methanol with 2M H2SO4 and 2M HCl. After dissolving, the reaction vessel was cooled in an ice bath to facilitate the precipitation of

the SNAP crystals. After 6 hours the crystals were collected via vacuum filtration and dried overnight. For the entire duration of the experiment the solution and crystals were shielded from light (to avoid activation of NO release with light as a stimulant).

Electrospinning of Fibers

240mg PLA is dissolved in 1.6mL THF at 100℃. The blank sample PLA was mixed with 0.4mL DMF, and the other three samples PLA/HN, PLA/SN, and PLA/SN/HN was separately mixed with 0.4mL DMF with 10wt% of honey, 10wt% of SNAP, and 10wt% of both honey and SNAP respectively. The mixture solution was loaded to the syringe with an 18-gauge electrospun needle. The syringe was put on a pump with a certain pushing rate at 1.2mL/h, the distance between the top of the needle to the aluminum foil on the collector was 15cm, and the applied voltage was 15kV. After the electrospinning, the fibrous mask was kept in a vacuum desiccator overnight to evaporate the solvent, then it was kept in a -20℃ freezer before use.

Scanning Electron Microscopy (SEM)

To examine the fiber morphology, uniformity, and size, SEM (FEI Teneo, FEI Company, Hillsboro, Oregon) was used with a 5.00 kV accelerating voltage. Prior to inspection, all samples were coated using a Leica Sputter Coater (Leica Microsystems, Wetzlar, Germany) with gold-palladium (10 nm thickness). ImageJ was used to analyze the fiber diameter after imaging.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of the nanofibers (in the form of KBr pellets) was analyzed using a Nicolet 6700 spectrometer (Thermo Electron Corporation, Madison, WI). For

each measurement, the spectra were obtained from 128 scans with a resolution of 4 cm^{-1} over the wavenumber range of 4000−400 cm^{-1}.

Tensile Testing

Mechanical properties of the fabricated nanofibers were evaluated using an Autograph AGS-X Series Precision Universal Tensile Tester (Shimadzu, Kyoto, Japan). Nanofibers were cut into a T-bar shape with a uniform length of 22 mm. Samples were fixed between two grips and pulled at a rate of 10 mm/min to attain stress/strain behavior. All testing was completed at room temperature.

Water Contact Angle (WCA)

Static contact angle measurements were carried out using a KRÜSS DA 100 drop shape analyzer (KRÜSS-Scientific; Matthews, NC) on 2 cm long square cutouts secured on a glass slide in a manner that ensured a flat surface. Approximately 5 µL droplets of deionized water was dispensed onto the nanofiber substrates with static contact angles measured from still frames using the sessile drop approximation. The average of five measurements spread across the surface of the film was recorded.

Equilibrium Water Absorption Percentage

The equilibrium water absorption ability was tested by submerging the nanofiber in DI water and incubated for 24h at 37℃. The dry weight of the sample was measured before, and the wet weight was measured after it was taken out from the water and gently and had the surface liquid absorbed with a Kimwipe. The equilibrium water absorption percentage was calculated using the Eq. 1 (W = Weight/mg):

$$\%\ equilibrium\ water\ absorption = \frac{Wwet - Wdry}{Wdry} \times 100\% \qquad (Eq.1)$$

Water Vapor Transmission Rate

Water vapor transmission rate (WVTR) of the mats was determined according to the following method [18]. A container was filled with 20 mg of deionized water and fibrous mats were used to fully cap the exposed area of the container and then tightened. The assembly was placed in a desiccator, containing anhydrous silica gel with relative humidity <5% at T=37C. The loss in weight of water was measured 8 times at time intervals of 1 h. Weight changes of the containers were plotted against time. WVTR was calculated using the Eq. 2:

$$WVTR = \frac{Slope \times 24}{A} \left[\frac{g}{m^2 \times day}\right] \qquad (Eq. 2)$$

Where A is the vapor exchange area being tested.

NO Release Measurements

NO release was recorded using a Sievers Chemiluminescence NOA 280i (Boulder, CO). The samples were wrapped in a dampened Kimwipe and suspended in a humid sample holder to simulate physiological conditions. It was maintained at 37 °C in a dark reaction vessel to prevent any light catalysis with 3 mL of PBS buffer solution (pH = 7.4) with EDTA in the bottom. EDTA was added to prohibit any metal ion activity in the buffer from interacting with the samples. To measure the NO levels released from each sample, nitric oxide was constantly swept from the headspace of the chamber and purged from the buffer solution by a bubbler and nitrogen sweep gas at 200 mL min^{-1} into the chemiluminescence detection chamber. In the chamber, NO reacts with ozone (O_3) to produce nitrogen dioxide (NO_2*) at an excited state. The excited nitrogen dioxide decays and emits a photon used to detect the original concentration of NO released measured in ppb. After taking into consideration the NOA constant (mol ppb^{-1} s^{-1}) and

the surface area of the sample, the data was converted to surface flux ($\times 10^{-10}$ mol cm^{-2} min^{-1}). Each sample was measured at 1, 4, 8, 12, 24 hours.

SNAP Leaching

The weight percentage of SNAP leaching from the PLA/SN sample was measured under physiological conditions for over the course of 24 h. The samples were soaked in 2 mL of PBS (pH = 7.4) and incubated at 37 °C at several time points in the 24 h time span. Absorbance was measured at 340 nm with a Thermo Scientific Genysis 10S UV–vis Spectrophotometer (UV–vis), which corresponds to the presence of the S-nitroso group of the SNAP molecule [19]. PBS was used as a blank control. The concentration of SNAP leaching was calculated from a calibration curve based on known SNAP concentrations dissolved in a PBS solution.

Antimicrobial Evaluation

In Vitro Bacterial Adhesion Assay

To study the antimicrobial activity of the nanofibers, a 24 h *in vitro* bacterial adhesion assay was performed according to a previously established protocol.[20] Nanofibers were tested against *S. aureus,* ATCC 6538. An isolated strain of *S. aureus* was inoculated in LB broth for 14 h at 37 °C at 130 rpm. After inoculation, the bacterial solution was centrifuged at 2500 rpm for 7.5 min and washed with sterile phosphate buffered saline (PBS, pH 7.4). The solution was again centrifuged at 2500 rpm for 7.5 min and resuspended in PBS. The bacterial solution was subsequently diluted to achieve a concentration of ~10^6 CFU per mL. Nanofibers were massed prior to experimentation. Nanofibers (n=3) were placed in a 24-well plate and immersed in 1 mL of the diluted bacterial solution. The plate was kept at 37 °C at 130 rpm for 24 h. After incubation, the

samples were removed and gently washed with sterile PBS to remove any loosely attached bacteria. The samples were then placed in separate 15 mL falcon tubes with 1 mL of PBS and homogenized for 60 s at 25,000 rpm and vortexed for 30 s. The resulting solution was serially diluted and plated on LB agar plates. Plates were stored at 37 °C for 24 h. After incubation, colony-forming units (CFUs) were counted to measure the number of viable bacteria per mg of nanofibers. Each nanofiber group was compared to control PLA fibers to determine the percentage reduction in adhered bacterial viability according to the following Eq. 3 (C = CFUs/mg):

$$\%\ reduction\ in\ adhered\ bacterial\ viability = \frac{C_{control} - C_{test}}{C_{control}}\ x\ 100 \qquad (Eq. 3)$$

Live/dead Staining

An isolated colony of *S. aureus* was inoculated in LB broth for 14 h at 37 °C at 130 rpm. The solution was subsequently centrifuged at 2500 rpm for 7.5 min, rinsed with sterile PBS, and centrifuged again. After washing, the bacterial solution was resuspended in PBS to achieve a final concentration ~10^6 CFUs per mL. Nanofibers were electrospun onto a glass slide, and 100 µl of the bacterial solution was used to cover the nanofibers. The nanofibers were incubated at 37 °C for 2 h. A live/dead dye was prepared by combining 2 µl of propidium iodide and 1 µl of SYTO9 into sterile DI water. After the incubation period was over, 30 µl of the prepared dye was added, and the nanofibers were stored in the dark for 15 min at room temperature. Using an EVOS FL Cell Imaging System (AMG, Mill Creek, WA), each nanofiber was imaged using different fluorescent channels.

Biocompatibility of the Nanofibers

Cell Culture

3T3 Mouse fibroblast cells (ATCC 1658) were cultured in 75 cm^2 T-flasks with Dulbecco's Modification of Eagles Medium (DMEM) supplemented by 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO2. The culture medium was replenished with fresh media every 2 days until cells were 90% confluent. Thereafter, the cells were detached from the T-flask surface by enzymatically degrading their extracellular matrix layer by treating them with 0.18% trypsin and 5 mM EDTA for 5 min.

Cell Viability

The effect of leach outs from the nanofibers were tested against fibroblast cell proliferation in DMEM medium. The study was carried out for 24 h in 5% CO2, 37 °C. Around 5000 cells/mL were seeded in a cell culture grade 96 well plate and incubated for 24 h in a humidified incubator with 5% CO2. The manufacturer's protocol (Sigma-Aldrich) was followed to perform the cytocompatibility test using a CCK-8 kit on the mouse fibroblast cells. After 24 h of cell culture incubation in a 96-well plate, 10 μL of the leachates (1mg/mL) from nanofibers was added (n = 7) to the cells. The cells were allowed to respond to the leachates during a separate 24 h incubation period inside a cell culture incubator at physiological temperature. After 24 h, 10 μL of the CCK-8 solution was added to the resulting solution and incubated for 4 h. During this time, dehydrogenase enzymes from live cells acted on the CCK-8 solution, converting it to an orange product, formazan, measurable at 450 nm. A standard curve was obtained for the number of viable cells corresponding to each O. D. Number of viable cells were calculated from the absorbance, which was measured after exposure to the extracts/leachates for 24 h. The relative viability (%) of the cells in response to

nanofibers leachates was reported relative to the number of viable cells in the control wells (without leachate exposure) using Eq. 4:

$$\%\ viable\ cells\ relative\ to\ control = \frac{number\ of\ viable\ cells\ in\ test\ samples}{number\ of\ viable\ cells\ in\ control\ samples} \times 100 \qquad (Eq.4)$$

Cell Adhesion

The prepared nanofibers were first cut into round disks (with a diameter of 6mm and weight of around 5 mg) and sterilized by UV radiation for 2 h on each side prior to cell seeding. In order to study the cell adhesion on the fabricated mats, fibroblasts (10000 cells/cm^2) were seeded on each sterilized sample and kept for 1 day in an incubator (T=37 °C, 5% CO2). Subsequently, the culture medium was removed, and the cells were fixed with glutaraldehyde solution (4% v/v) in PBS at 4 °C for 30 min. The samples were then dehydrated through a series of graded ethanol solutions and then prepared for imaging with SEM.

Statistical analysis

All data were calculated as mean ± standard deviation. Population standard deviation calculation was used in bacterial and cytocompatibility analyses. Student's t-test with unequal variance was used to calculate p values, which was used to determine a significant difference between values.

2.3 Results & Discussion

SEM

A series of different honey percentages (5, 10, and 15%) was used to test the effects on the fiber structure, to maximize the concentration of honey without negatively

impacting the surface morphology of the fibers, we decided to combine 10 % honey with 10% SNAP. The SEM images of the electrospun fibers are shown in **Figure 1**. which were used to calculate the average fiber diameter. Honey concentrations exceeding 10% resulted in increased surface roughness. This is caused by the high concentration of honey, so the viscosity of the solution increases, making it more difficult to be sprayed.

The average diameter was calculated using ImageJ software and is shown in **Table 1**. No significant change on the diameter was found between any of the sample types. Porosity and fiber diameter are important factors when relating to cell migration and adhesion, studies also show that inflammation-related processes can be reduced on a less flat (non-nanostructure) surface [21]. 10% was chosen as the main sample to maximize honey content, while not negatively impacting surface morphology.

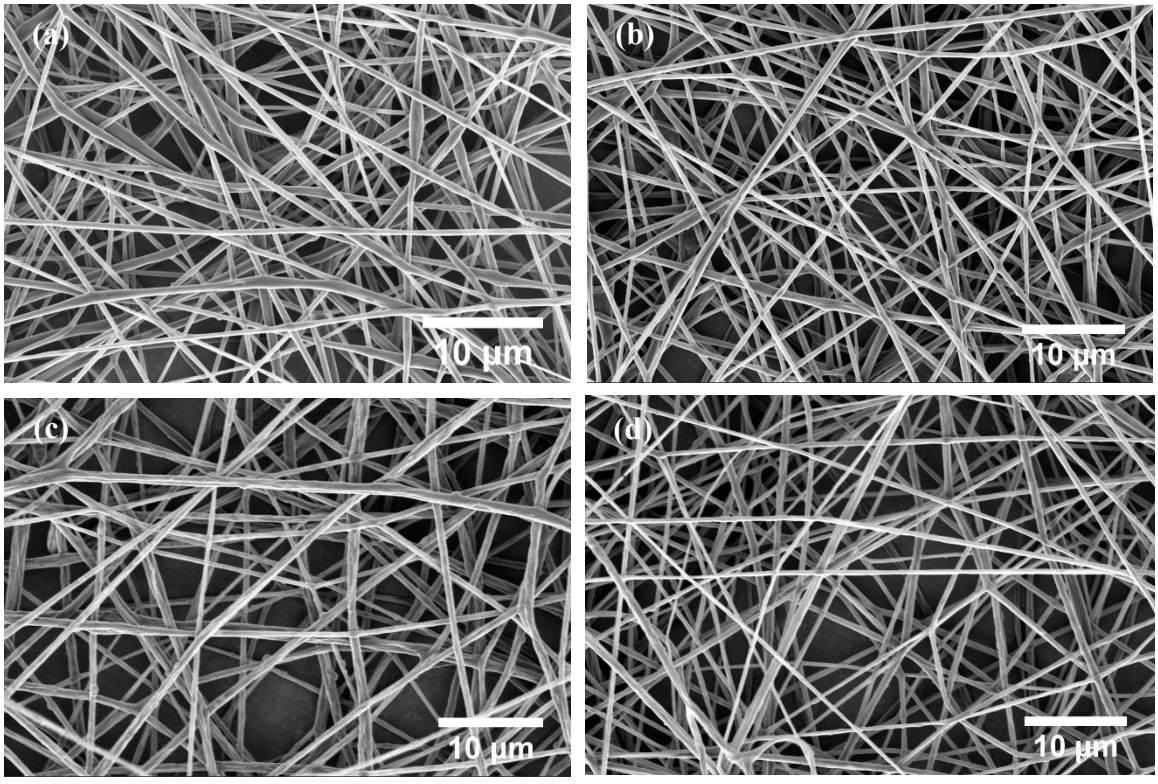

Figure 1 – SEM image of PLA nanofibers with different weight percentages of honey: (a) 5%, (b) 10%, (c) 15% and (d) 10% honey with SNAP.

Table 1 – Average diameter of PLA nanofibers with different wight percentage honey: 5%, 10%, 15% and 10% honey with SNAP. Data is reported in mean ± standard deviation. No significant difference was found between any of the sample types ($p > 0.05$).

Honey percentage	**5%**	**10%**	**15%**	**10% with 10% SNAP**
Diameter/nm	766.48 ± 110.42	766.54 ± 145.57	784.21 ± 168.43	624.92 ± 137.69

Fourier Transform Infrared Spectroscopy

FTIR was utilized to verify the interactions of different components and chemical composition of the nanofibers. **Figure 2** shows the FTIR spectra of Honey, PLA, PLA/HN, PLA/SN and PLA/SN/HN nanofibers. Honey shows characteristic bands at 2945, 1651, and 1069 cm^{-1}, related to C-H, C=O, and C-O stretching vibrations respectively, which is consistent with the previous reports [22]. PLA exhibits characteristic stretching frequencies for C=O, –CH3 asymmetric, –CH3 symmetric, and C–O, at 1753, 2996, 2942 and 1080 cm^{-1}, respectively. Bending frequencies for –CH3 asymmetric and –CH3 symmetric were also observed at 1457 and 1363 cm^{-1} [23]. Addition of either SNAP or HN alone did not cause any noticeable change in the intensity or shift in the peaks of PLA suggesting that there is no new bond formed or strong chemical interaction occurring within the nanofiber composites [24]. However, co-addition of HN and SN causes a shift in C=O bond from 1753 to 1737 cm^{-1} and C-H bond from 2996 to 2971 cm^{-1} which probably indicates the formation of physical hydrogen bonds between SNAP and HN.

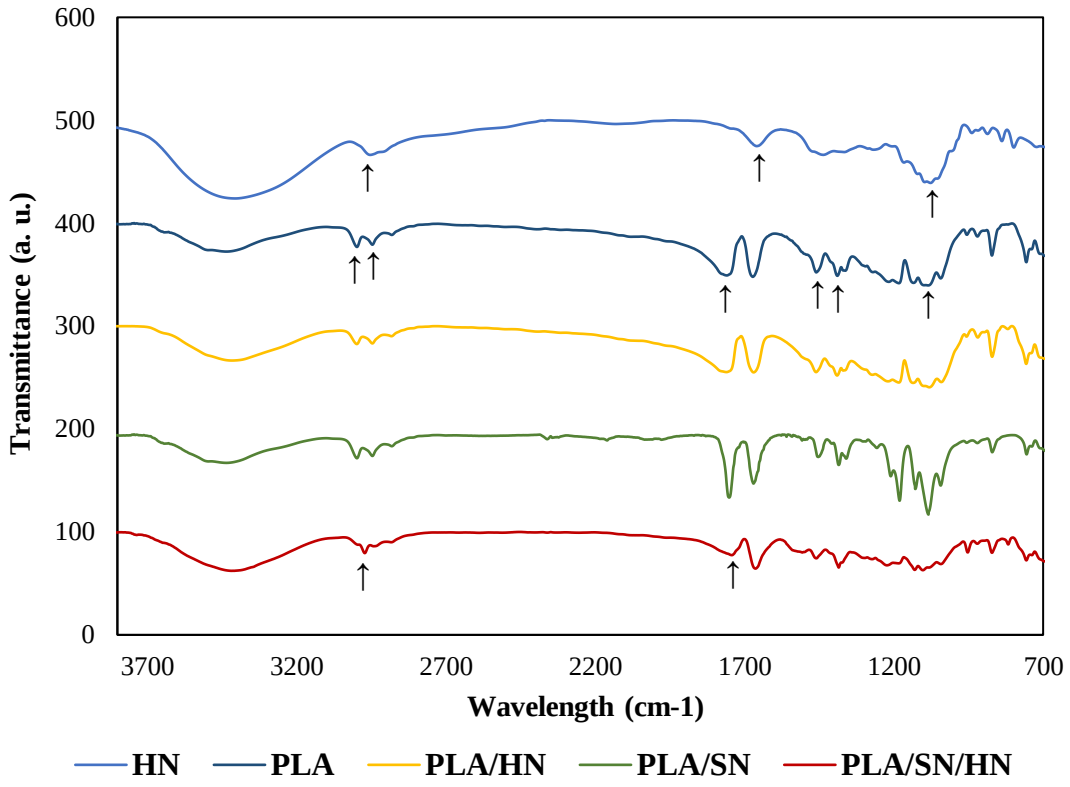

Figure 2 – FTIR spectra of Honey, PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers.

Characterization of Mechanical Properties

Mechanical reliability is crucial for nanofibers used for biomedical applications including wound healing, tissue engineering, and vascular grafts. To determine the mechanical properties of the fabricated materials, the tensile strength of the PLA/SN/HN nanofibers was investigated (n=5) and compared to control fibers (PLA, PLA/HN, and PLA/SN). **Figure 3** shows the stress/strain behavior for each of the fiber types. No statistical difference was found between the ultimate tensile strength, elongation at break, or Young's modulus between each of the sample types (**Table 2**) ($p > 0.05$). Previous studies which evaluated nanofibers for biomedical applications showed very similar

stress/strain profiles and mechanical properties, indicating that the nanofibers for this study have suitable elastic and tensile characteristics.[25-27]

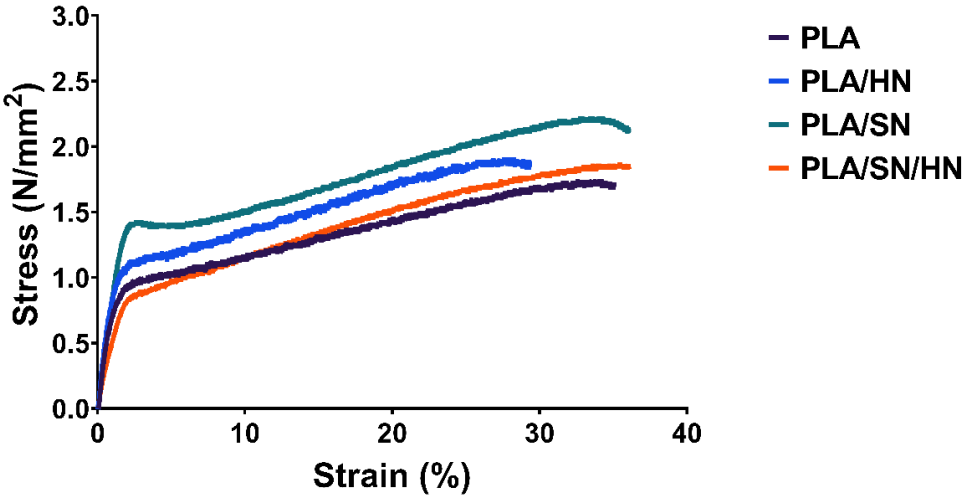

Figure 3 – Stress/strain curves of PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers.

Table 2 – Mechanical properties of PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers. Data is reported in mean ± standard deviation. No significant difference was found between any of the sample types ($p > 0.05$).

	Ultimate Tensile Strength (N/mm^2)	Elongation at Break (%)	Young's Modulus (N/mm^2)
PLA	2.0 ± 0.8	35.7 ± 9.3	78.1 ± 24.5
PLA/HN	2.0 ± 0.7	29.0 ± 4.4	82.5 ± 28.4
PLA/SN	2.6 ± 0.4	32.3 ± 4.3	64.3 ± 12.1
PLA/SN/HN	2.2 ± 0.6	33.0 ± 3.5	66.7 ± 25.7

Water Contact Angle

The water contact angle was measured to determine the hydrophilicity of the PLA material before and after loading with honey or SNAP, but it is not possible to use fibers due to the rapid absorption of water into the fibrous network. Instead of using fibers, the films of each sample type were made with the same polymer solution. Results are shown

in **Table 3**. No significant difference in the contact angle measurements was seen with the presence of honey. By blending SNAP, there was a significant decrease in the contact angle, representing that hydrophilic nature had increased. This indicates that PLA/SN/NH fiber has enhanced wettability, which allows for better fibroblast attachment [28, 29].

Table 3 – Water contact angle of PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers (n=5). Data is reported in mean ± standard deviation. Both PLA/SN and PLA/SN/HN have significant decrease compare to PLA and PLA/HN ($p<0.005$).

	PLA	**PLA/HN**	**PLA/SN**	**PLA/SN/HN**
WCA (°)	77.65 ± 1.67	78.60 ± 1.14	72.77 ± 2.48	71.23 ± 3.07

Equilibrium water absorption

The material's water absorption capacity modulates its ability to absorb wound exudate and its compatibility to the moist environment of the wound site. The water within the dressing not only increases the rate of epithelial formation, but also promotes healing through prevention of tissue dehydration and cell death, accelerated angiogenesis [30]. The equilibrium water absorption percentage is shown in **Table 4**. Honey and SNAP both can substantially increase the water absorption of PLA. Under the synergistic action, PLA/SN/HN has a highly significant, 50-fold increase compared to the control ($p=0.0001$).

Table 4 – Equilibrium water absorption percentage of PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers (n=3). Data is reported in mean ± standard deviation. All three other samples have highly significant increase compare to PLA control ($p<0.001$).

	PLA	**PLA/HN**	**PLA/SN**	**PLA/SN/HN**
Water absorption %	7.20 ± 2.58	258.66 ± 33.33	274.67 ± 66.52	378.96 ± 17.12

Water Vapor Transmission Rate

The water vapor transmission rate (WVTR) was measured to evaluate the moisture permeability of the nanofibrous scaffolds. WVTR is a critical factor for scaffolds used in wound healing and skin tissue engineering applications. It is suggested that an optimum amount of WVTR (2000–2500 g m^{-2} day^{-1}) is required to balance the moist environment at the wound site for improved wound healing and inhibition of secondary bacterial infections [31-33]. As can be seen in **Table 5**, all prepared nanofibers in this study had similar WVTR ranging from 2700 to 3000 g m^{-2} day^{-1} which is close to the ideal WVTR values for wound dressings. Therefore, it can be inferred that the developed PLA/SN/HN nanofibers are suitable for treatment of high exudate wounds.

Table 5 – Water vapor transmission rate of PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers (n=3). Data is reported in mean ± standard deviation. No significant difference was found between any of the sample types ($p > 0.05$).

	PLA	**PLA/HN**	**PLA/SN**	**PLA/SN/HN**
WVTR(g/m^2day)	2859.4 ± 49.46	2970.3 ± 82.32	2820.8 ± 45.31	2875.3 ± 47.52

NO Release Characterization

Nitric oxide release was examined for both the PLA/SN and PLA/SN/HN using a Sievers Chemiluminescence Nitric Oxide Analyzer (Figure 4A). The detection of NO using chemiluminescence is the "gold standard" in quantifying NO release due to its selectivity for the gaseous NO released, rather than the nitrates and nitrites that may also be present. **Figure 4** illustrates the NO release from the PLA/SN and PLA/SN/HN fibers.

During the first 6 h of release, PLA/SN shows a higher release than PLA/SN/HN. After 7 h, however, PLA/SN/HN fibers have a higher release rate. This observation might be caused by the blending of honey which prevents the decomposition of SNAP to a certain extent. As the honey dissolves and is released, the NO release rate is also accelerated. Therefore, honey has a controlling effect on NO release and can prevent the initial burst release. The NO flux was evaluated for 48 h. After 24 h incubation, both samples release at very low rates, which did not exceed more than 0.1 ($\times 10^{-10}$ mol min^{-1} mg^{-1}). However, even low levels of NO release have shown to be effective in reducing the viability of bacteria [34]

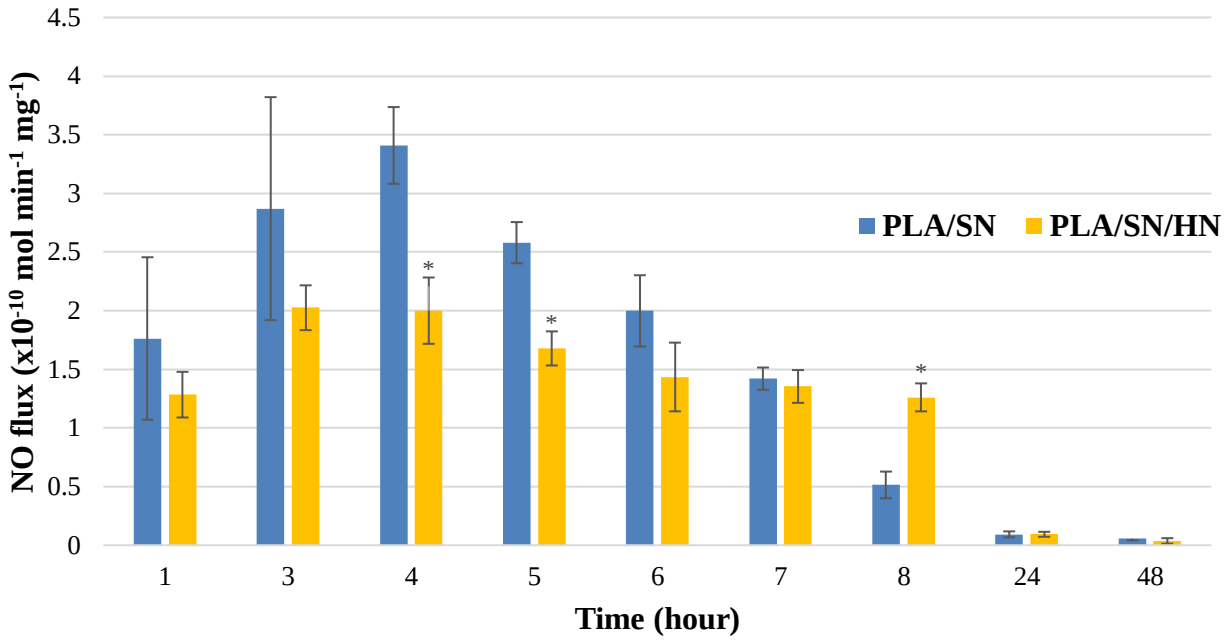

Figure 4 – The nitric oxide release profiles for PLA/SN and PLA/SN/HN nanofibers over 48 h at 37 °C. (* indicate $p < 0.05$ compared to PLA/SN at the same time period, n=3).

SNAP Leaching

Leaching of the NO donor from the material can have disadvantageous effects on the longevity of the release characteristics and could result in a nonlocalized release [20]. **Figure 5** shows the SNAP percentage remained in the fiber after soaking. 94.83 ± 0.82% SNAP was remained at the end of 24 h period, suggesting that most of the NO donors could be well stored in PLA fibers.

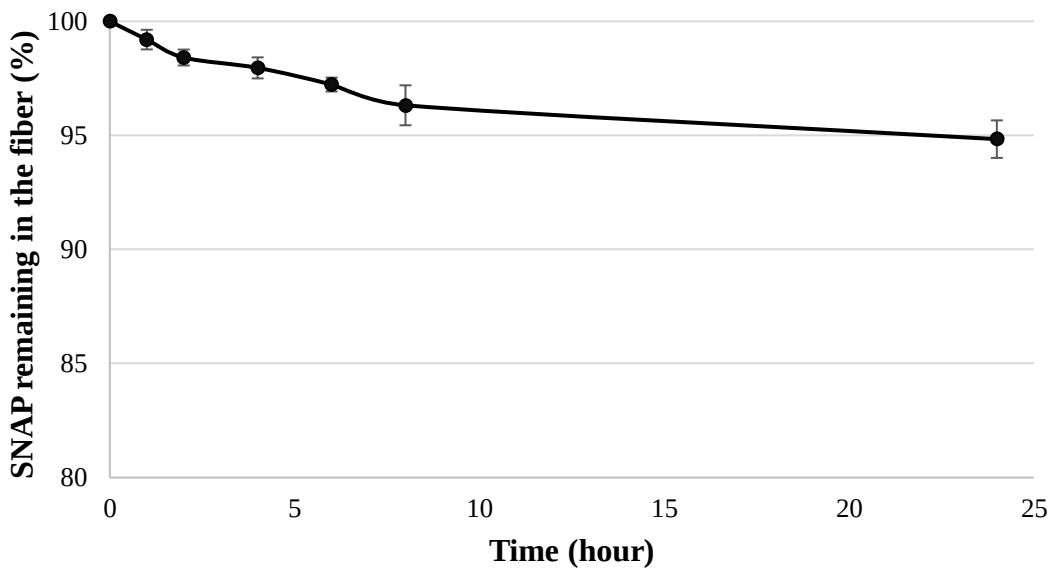

Figure 5 – SNAP percentage remaining in PLA/SN nanofibers during 24 h time period (n=3).

In vitro Evaluation of Antimicrobial Efficacy

Nanofibers have recently been utilized for many biomedical applications including tissue engineering, vascular grafts, medical device coatings, and wound healing treatments. However, the efficacy of these materials is largely still burdened by infection. Therefore, nanofibers which include robust antimicrobial agents to deter the growth and colonization of pathogens are imperative for nanofibers used in biomedical therapies. To determine the antimicrobial activity of the PLA/SN/HN fibers, a 24 h bacterial adhesion assay was performed against *S. aureus*, a common pathogen related to nosocomial infections (**Figure 6**). After 24 h exposure to S. aureus, PLA/HN, PLA/SN, and PLA/SN/HN fibers significantly reduced bacterial viability by 88.4 ± 2.8%, 93.4 ± 2.4%, and 96.4 ± 0.6%, respectively ($p < 0.05$). NO-releasing materials previously have similarly demonstrated broad-spectrum antibacterial effects capable of >90% reduction in

bacterial viability.[35-37] This can be attributed to the potent antimicrobial activity of NO used by macrophages *in vivo*, leading to lipid oxidation, DNA deamination, and protein modifications.[38] Similarly, honey has been reported to have antibacterial activity due several properties including hydrogen peroxide production, low water activity, and acidity.[39]

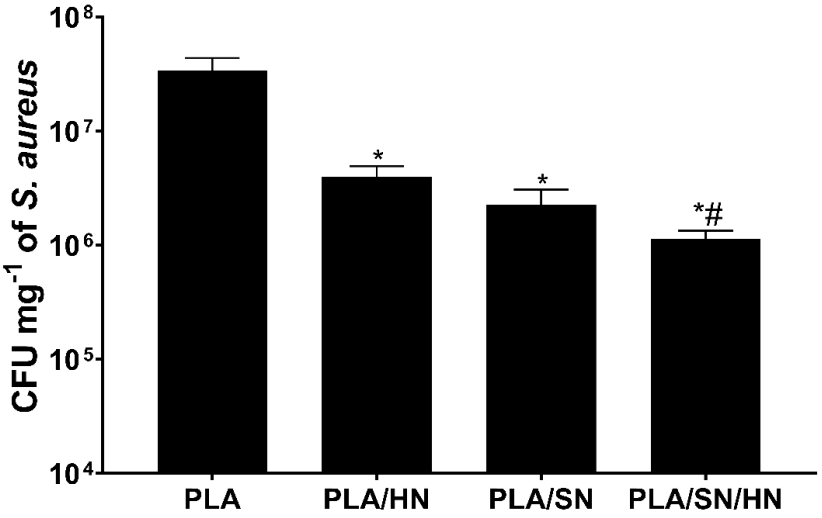

Figure 6 – Antimicrobial activity of PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers against *S. aureus*. PLA/SN/HN nanofibers best reduced the viability of adhered *S. aureus*. (* and # indicate $p < 0.05$ compared to PLA and PLA/HN, respectively).

To observe the viability of exposed *S. aureus* as a function of membrane integrity, a live/dead bacterial viability assay kit was used (**Figure 7**). All cells were stained green (SYTO9 – nonexclusive membrane dye) and compromised bacterial cell membranes stained red, indicating loss of viability. Live/dead quantification using Fiji indicated that *S. aureus* cells exposed to control PLA fibers retained 96.6 ± 2.6% viability, while only

40.7 ± 13.6%, 19.9 ± 14.4%, and 14.8 ± 10.0% of *S. aureus* cells exposed to PLA/HN, PLA/SN, and PLA/SN/HN retained viability, respectively (**Figure 8**). This is consistent with the trend found in the 24 h colony-counting assay that the combination of SNAP and honey best reduced bacterial viability, providing a promising platform that reduces the risk of microbial infection for nanofiber-related biomedical applications.

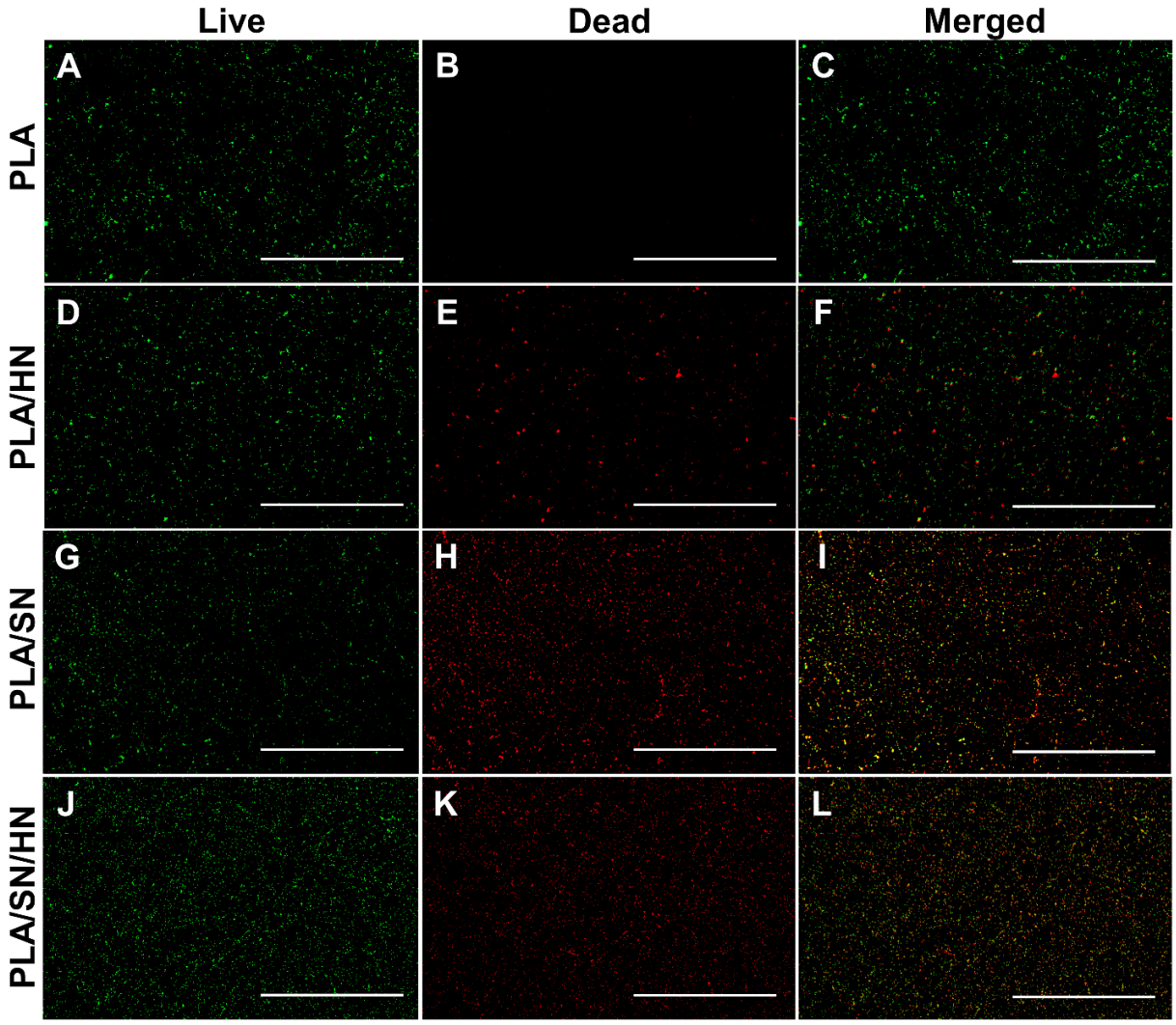

Figure 7 – Live/dead staining of *S. aureus* exposed to PLA (A-C), PLA/HN (D-F), PLA/SN (G-I), and PLA/SN/HN (J-L) nanofibers. White bar = 200 µm.

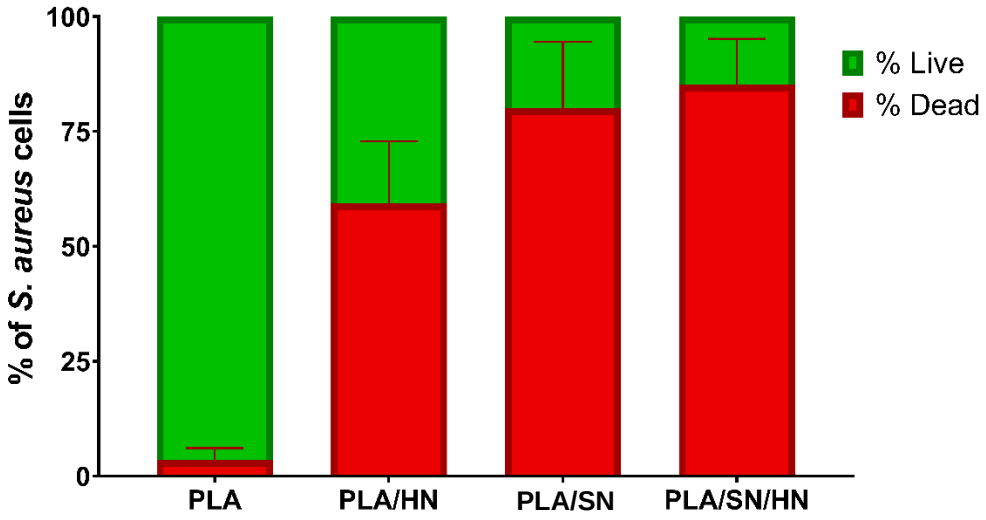

Figure 8 – Live/dead quantification of *S. aureus* exposed to PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers. PLA/SN/HN showed the highest percentage of dead bacteria on the nanofibers.

Biocompatibility of the Nanofibers

Biocompatibility is a primary requirement for biomaterials used in wound healing process. The evaluation of the *in vitro* cytotoxicity of a biomaterial is the initial step in a biocompatibility study [40]. In the present study, the biocompatibility of the prepared nanofibers was studied both qualitatively and quantitatively by analyzing the morphology of 3T3 fibroblast cells adhered to the nanofibers and measuring their viability after being cultured in the leachate medium of nanofibers. CCK-8 dye-based cell viability assay was performed on mouse fibroblast cells using 24 h leachates from different nanofibers. Results for the test (**Figure 9**) indicated that all samples possessed levels of fibroblast cell viability similar (>90% cell viability) to those of the control (cells in the cell culture well without any material). However, the PLA/SN/HN sample presented a significantly higher cell viability compared to pure PLA nanofibers which suggests an excellent

biocompatibility is obtained by addition of both HN and SN ($p<0.05$). To further confirm the biocompatibility of PLA/SN/HN composite fibrous matrices, the morphology of 3T3 fibroblast cells grown on the nanofibers were observed by SEM after 1 day of culture. As can be seen in **Figure 10**, cells had a strong interaction with all nanofibers and exhibited extended morphologies. In some areas cell infiltration into the nanofibers is observed which is a critical parameter for the successful development of 3D matrices for tissue engineering [41]. After addition of HN and SN a clear increase in cell-cell interactions and formation of cell sheets on the nanofibers can be seen. Moreover, a higher number of cell attachment and distribution was observed on PLA/SN, PLA/HN, and PLA/SN/HN nanofibers as compared to pure PLA nanofibers. These observations suggest the incorporation of HN and SN could further improve the affinity of PLA nanofibers for cells. The enhanced cellular behavior on SN and HN containing nanofibers might be attributed to the higher water uptake of these nanofibers compared to PLA. It is previously reported that higher water uptake ability allows for absorption of more serum proteins and causes higher degradation rate leading to generation of a rougher surface and therefore results in improved cellular adhesion [42]. In addition to physical properties, cell-material interaction is also affected by the chemical composition of the material. Addition of HN and SNAP to PLA nanofibers, increases the amount of OH groups in the nanofibers structure which in turn can increase the cells affinity to the surface. Previous studies have suggested the need of an optimal density for hydroxyl groups to obtain good cell adhesion [42]. Overall, the desirable adhesion, proliferation, and infiltration of cells into the nanofibrous scaffolds of PLA/SN/HN, suggest their potential for various tissue engineering applications.

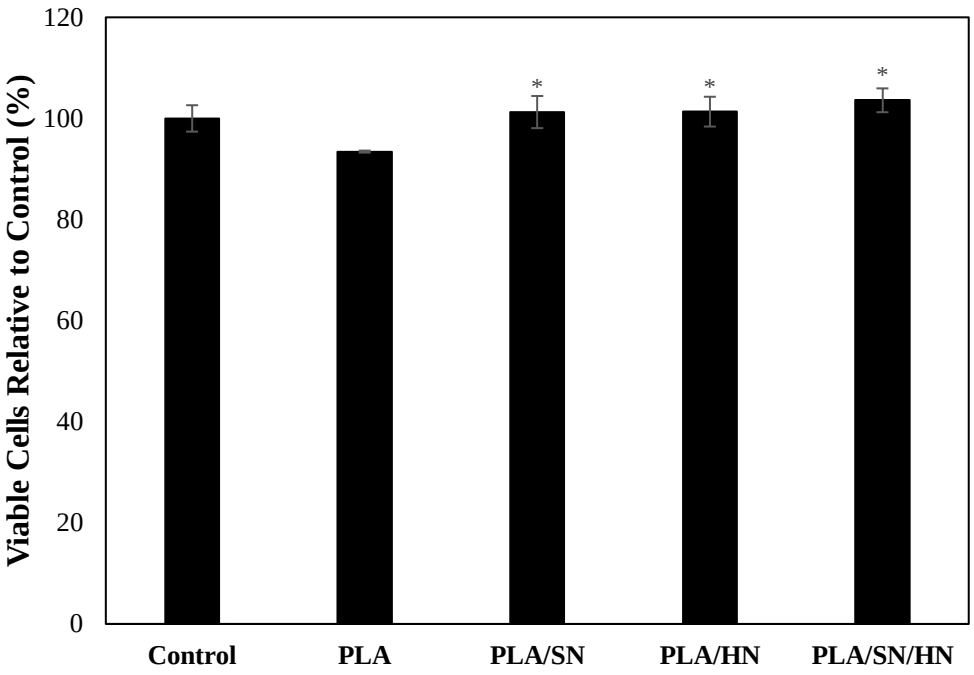

Figure 9 – CCK-8 dye-based cell viability assay on mouse fibroblast cells using 24 h leachates from PLA, PLA/HN, PLA/SN, and PLA/SN/HN nanofibers, the number of cells is represented as percentages relative to the control. (* indicate $p < 0.05$ compared to PLA).

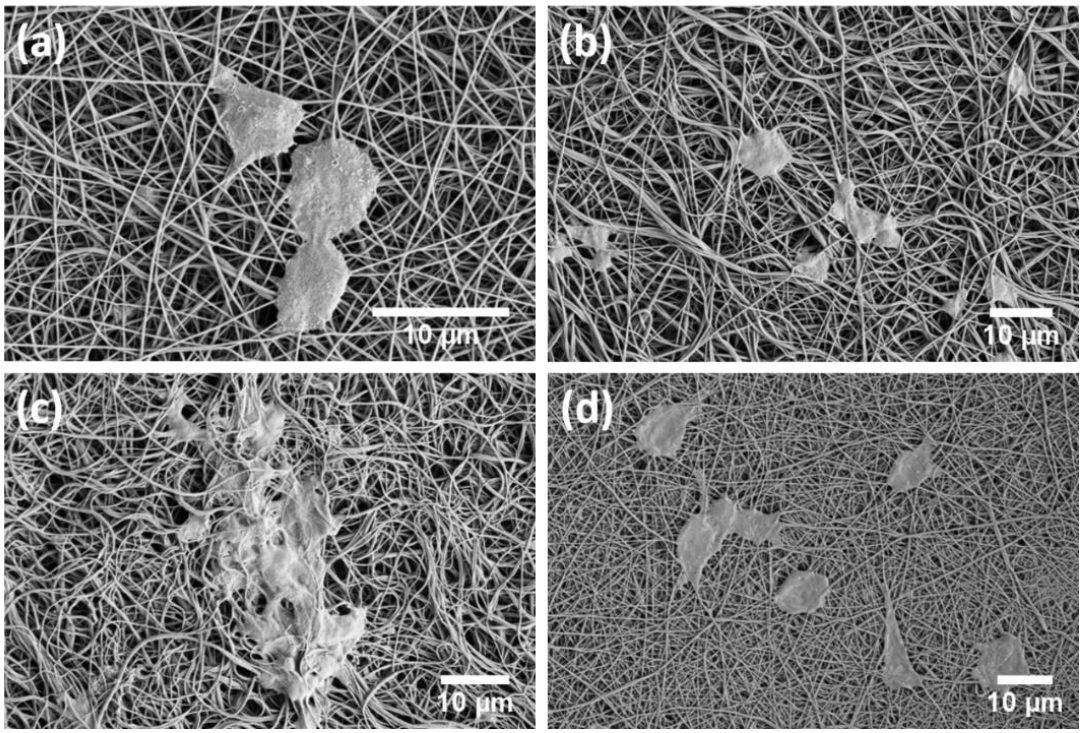

Figure 10 – SEM image of 1-day culture of 3T3 fibroblast cells grow on (a) PLA, (b) PLA/HN, (c) PLA/SN, and (d) PLA/SN/HN nanofibers.

2.4 Conclusions

In this study, a novel wound dressing is developed that exhibits excellent wound healing and wound protection properties. Electrospun PLA nanofibers provide a fibrous network with high surface area and porosity, which helps capture and remove exudate from the wound, while also maintaining a moist environment at the wounded area. The synergistic effects of honey and SNAP exhibit a 96.4 ± 0.6% bacterial killing effect, with an excellent biocompatibility. It was also observed that the porous fibers aided in the cellular adhesion of the wound healing process, acting as a scaffold to support cell attachment and proliferation.

This research provides preliminary work towards the development of a novel, multi-functional wound dressing. Further *in vivo* studies are needed to assess the full synergy of this material.

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CHAPTER 3

CONCLUSIONS AND FUTURE WORK

3.1 Conclusions

The preface in Chapter 1 gives a description of the processes involved wound healing and the possible bacterial infections that may occur. Furthermore, it provides a brief analysis of antibiotics and current existing healing methods. This subject has been researched and developed for many decades. Drawing on those strengths and weaknesses, this project is designed to develop a biocompatible and antibacterial electrospun wound dressing using honey and SNAP. This is described in detail in Chapter 2.

This study provides preliminary work towards the development of a novel, multi-functional wound dressing. The synergistic use of SNAP and honey strengthens the advantages of both, and PLA is not only a carrier but moreover a scaffold for cell growth and provides a great healing environment. The results have proved that this material has great antibacterial capability and biocompatibility, with excellent physical properties for wound sites. We hope that this material can be used in medical clinics in the future to solve problems such as wound infection and delayed healing.

3.2 Future Work

Before moving to clinical application, further characterization and testing will be needed. The next step in this project, is to use a mouse wound model to evaluate in vivo effectiveness of the fibers on wound closure and healing. Expanding to in vivo studies

will provide a deeper understanding of how other aspects of honey and SNAP will affect the wound healing process. Together, they may have a profound influence to promote the healing process of the human body, affecting the response of factors such as inflammation factors and immune cells.

The animal model is designed to apply this material to the open wounds of mice, with a blank control group which will be dressed with saline solution only. The healing of the wound will be judged histopathologically by measuring the thickness of granulation tissue, epithelization from the periphery of the wound, and the size of the open wounds. It is expected that the PLA/SN/HN dressing will promote faster healing with a smaller size of wound. If the results are positive, the next step is conducting an animal model test that is more suitable for human skin, such as pigs. Future research and experimentation will be more detailed and specific, to aim for a certain or one kind of related type of wound. The ultimate goal of this project is for this novel material to be mass-produced and used in clinical treatment, it will be achieved through future research and improvement.

As a whole, this work is aimed to develop an effective medical tool which can help with wound related diseases. Furthermore, it hopes to provide a better understanding of the material development process and create new ideas for future work.