Doctoral Dissertation (Shinshu University)

Study on fabrication of bioactive polymeric fibers for wound care and bone regeneration.

創傷被覆材および骨再生治療効果を有した生体活性高分子繊維の作製に関する研究

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Abstract

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Over the past few decades, different spinning techniques have been explored for their abilities to fabricate complex fibrous structures having great potential for biomedical applications. The biocompatible and biodegradable systems made of polymeric fibers are of great interest in biomedicine, particularly in wound healing due to their high surface-to-volume ratio, interconnected structure, open pore structure, and tunable surface and mechanical properties, apart from utilizing inherent properties of the polymers used in the production of the fibrous scaffolds. The modern fiber spinning techniques also allow us to incorporate bioactive components within their matrix, making it possible to deliver them topically to the targeted areas, which can be beneficial for improving the healing process.

Bio-textiles based on natural, regenerative, and synthetic fibers are highly desirable in regenerative applications. They are designed for their use in a specific biological environment, where they are evaluated for their interaction with the cells and other biological fluids and measured as biocompatibility and biostability.

Electrospinning is a versatile technique for development of a wide variety of nanofibers. It is employed for developing electrospun fibers for a wide range of applications from separations membranes to tissue engineering. Electrospinning has improved the qualities of the pristine polymers by enabling the incorporation of nanoparticles and other bioactive compounds, and this have been particularly important for the tissue engineering applications, drug delivery, and wound care; the three themes of regenerative medicines. Nanofibers are an important material for regenerative medicine as they can mimic the morphology of the extracellular matrix and can support and hold the growth of cells and tissues within the body. Nanofiber's functionality can be extended by incorporating them with other bioactive compounds and materials.

In the present work we explored electrospinning technique to develop bioactive compounds incorporated polymeric nanofibers for wound care and bone tissue engineering. We evaluated their physicochemical and biological properties as potential scaffolds for regenerative applications.

Chapter 1 provides an overview of the thesis book, including previous literature about fibrous scaffolds for regenerative medicine and related techniques used to manufacture fibrous scaffolds for tissue engineering.

Chapter 2 concludes a study on Manuka honey incorporated cellulose acetate nanofibrous mats as a potential wound dressing. Here we report the fabrication of cellulose acetate (CA) – manuka honey (MH) composite nanofibrous mats as a biocompatible and antimicrobial wound dressing. CA mats with different quantities of MH were developed by electrospinning. The ATR-FTIR spectra confirm the inclusion of MH in the composite CA-MH nanofibrous mats. The fibers were continuous and bead-free with good mechanical properties. The fiber diameter increased with an

increase in MH content. Inclusion of MH in the electrospun composite CA-MH nanofibrous mats shows high efficacy in preventing bacterial growth on the wound surface. The MH-loaded CA nanofiber mats showed good antioxidant abilities. In contrast, the ability to free radicalize the DPPH depended on the factors of MH content in the fiber and the time of immersion in the DPPH solution. Besides, the nanofibrous mat's high porosity (85 - 90 %) and WVTR values of 2600 to 1950 g/m2/day, suitable for wound breathability, and the mats show high cytocompatibility to NIH3T3 cell line in in-vitro testing, proving to be effective for promoting wound healing.

Chapter 3 concludes a study on essential oil incorporated cellulose acetate nanofibrous matrix for antibacterial, and antioxidant wound dressing. Blumea balsamifera oil-loaded cellulose acetate nanofiber mats were prepared by electrospinning. The inclusion of blumea oil increased the nanofiber diameter. FTIR spectra confirm the addition of blumea oil in the nanofiber mats. The XRD pattern suggests that the inclusion of blumea oil has caused a misalignment in the polymer chains of the cellulose acetate. Thus, a decrease in the tensile strength was observed for the blumea oil-loaded nanofibers. The increase in fiber diameter causes a reduction in the porosity of the nanofiber mats. The blumea oil-loaded nanofiber mats showed antibacterial efficacy against Escherichia coli and Staphylococcus aureus. The blumea oil showed antioxidant abilities against the DPPH solution. MVTR of the neat and blumea oil-loaded nanofiber mats were in the range of 2450 – 1750 g/m2/day, which is adequate for the transport of air and moisture from the wound surface. Blumea oil-loaded mats showed good cell viability ~92% for NIH 3T3 cells in more extended incubation periods. A biphasic release profile was obtained, and the release followed the first-order kinetics depending upon the highest value of the coefficient of correlation R2 (88.6 %).

Chapter 4 concludes a study on illite clay based biocompatible nanofibrous scaffold for bone tissue engineering. Osteoconductive bone tissue scaffolds are of particular interest. Clay composed of aluminosilicate can play an essential role in bone repair and regeneration, enhancing osteoblastic differentiation and apatite deposition. Current research focuses on the fabrication of illite clay incorporated polycaprolactone/zein electrospun scaffolds. The prepared scaffolds by varying concentrations of Illite were characterized physiochemically and biologically. The composite scaffolds were analyzed for surface morphology, mechanical properties, thermal characteristic, crystal structure, surface wettability, and chemical modification. Biocompatibility of the composite scaffold was checked against the MC3T3-e1 cell line as a model. The biomineral apatite deposition was determined by incubating the scaffolds in simulated body fluid. The alkaline phosphatase staining evaluated osteoblastic differentiation. The results show that the clay composite scaffolds showed reduced fiber diameters and enhanced wettability. The addition of Illite improved the tensile strength and the Youngs Modulus of the composite scaffolds. The in vitro biomineralization of the composite scaffolds improved calcium deposition with the maximum Ca/P ratio of 1.55. The WST-1 assay and ALP staining show that the composite scaffolds have better cell viability and osteoblastic differentiation than the PCL/zein scaffolds. The overall results show that the clay-corn-caprolactone scaffold is a promising material for bone tissue engineering.

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Chapter 1

General Introduction

1.1 Background

Over the past few decades, the textile industry has undergone a considerable overhaul, from fulfilling the basic purposes such as clothing, aesthetics functions, and containers, to functional products like filters, structural reinforcement, biomedical, protection, automotive, agriculture and more. With the advent of these high-end products the emphasis on the performance of these product is ever increasing. Thus, resulting in a paradigm shift from passive textiles to more advanced active textiles. The purpose for the advance textile product is to be functionally engineered to suit a specific intended environment, and to be able to stimulate the surrounding elements beneficial for the targeted application.

Textile based fibrous assemblies have found great interest in the biomedical fields especially in the tissue engineering, drug delivery, and wound healing, the three divisions of regenerative medicine. The wound care sector is one of the most flourishing avenues in pharmaceuticals. The global wound care market accounts for USD 19.3 billion in 2021 and is projected to reach USD 27.8 billion by 2026 (MarketsandMarkets). The surge in the wound care demand is from both the acute wounds from surgeries and chronic wounds resulting from diabetic ulcer etc. Skin Injuries lays a major burden on the healthcare systems especially in the countries having old population. The skin is the largest organ in human body and serves as the first line of defense against the external hazards. Skin plays a major role in maintaining the moisture balance, temperature, and protection from the pathogens. Timely repair of the skin is essential for the survival of the living beings [1].

Bone tissue engineering is another division of the regenerative medicine and involves the understanding of bone structure, bone mechanics, and tissue formation as it aims to induce new functional bone tissues. The global tissue engineering market accumulates around USD 9.9 billion in 2019 and is expected to yield an annual growth rate of 14.2% from 2020 to 2027 (grandviewresearch). Bone and joint disorders are ever increasing especially in the population where the aging is coupled with increased obesity and poor physical activity. Engineered bone tissue are viewed as a potential alternative to the conventional use of bone grafts; autografts, and allografts; because of their ease of availability and potentially no disease transfer. The field of bone tissue engineering was initiated nearly 4 decades ago. The focus in developing scaffolds for bone tissues are to eliminate the current clinically related issues including i.e. donor site morbidity, limited availability, immune rejection, and pathogenic transfer. The bone tissue engineering paradigm highlights several key players: (1) a biocompatible scaffold that closely mimic the natural

bone tissue extracellular matrix, (2) osteogenic cells to lay down the bone matrix, (3) the stimulus to help direct the cells to the phenotypically desirable type, (4) sufficient vascularization to meet the growing tissue nutrients supply [2].

1.2 Nanofibers for biomedical and healthcare applications

Nanotechnology is considered as one of the most important parts of science and technology in the twenty first century that can be compared to the industrial revolution. Nanofibers are one of the interesting groups of nano materials with two dimensions in the sub-micron range and the third dimension significantly larger. Nanofibers are defined as structures with a diameter of less than 1 μm by the U.S. textile industry and Japanese and Korean strategic research initiatives, which is different from the National Science Foundation definition of nanotechnology, where the structure less than 0.1 μm are classified as nanomaterials [3].

Nanofibers have a lot of wonderful characteristics like high surface to volume ratio, ability to carry bioactive agents, surface functionalization properties, tunable mechanical properties, interconnected porosity, a wide range of biocompatible material selection, and ability to form hybrid structures. These extraordinary properties make them an ideal candidate for biomedical applications like tissue engineering[4], drug delivery[5], and wound care[6]. Tissue engineered scaffolds are considered as a satisfactory solution to aid the health quality of life for millions of patients worldwide [7].

Nanofibrous scaffolds provide many appropriate properties such as high porosity, large surface area, biocompatibility, intended mechanical properties, provide support and house cells required for tissue regeneration. The ability to choose from a wide variety of degradable and non-degradable materials give nanofibers a great flexibility over the scaffold intended application like control over the release of drug and other growth factors. The nanofibers provide the possibility to immobilize enzymes, growth factors, drugs, antibiotics, and other bioactive compounds both within and on the surface of the nanofibers. The ability of the nanofibers to mimic the morphology of the extracellular matrix with high interconnected porosity, ability to regulate moisture, and gas permeability protect wounds from exogenous infection. In traducing bioactive agents into nanofibers provide a great environment to treat infections in chronic wounds and diabetic ulcers [8].

1.3 Nanofiber fabrication techniques

Various mechanical, chemical, and electrostatic methods have been employed for the fabrication of nanofibers. Generally, the techniques for nanofiber manufacturing can be classified in two categories: (1) physical, chemical, and biological techniques; and (2) spinning and non-spinning techniques.

Physical nanofiber manufacturing techniques employ mechanical pressure, electrical energy, or high energy radiation to cause material melting, abrasion, evaporation, or condensation to form nanofibers. Common techniques include physical vapor deposition [9], laser ablation [10], and spinning fabrication techniques [11]. In these techniques the material in solid or liquid form goes to vapor phase and recondense to form nanofibers structures. Other physical techniques like ball milling, and high-pressure homogenization are used in the top-down methods to fabricate nanofibers. For example, the production of cellulose nanofibers from fibrillation of cellulose plant by grinding, and high-pressure homogenization [12].

Chemical method is based on a chemical reaction between two or more chemical species to form a nanofiber. The chemical reaction could be self-sustaining or can occur by an external factor including radiation, electrical energy, and thermal energy. Commonly employed methods include chemical vapor deposition [13], sol-gel process [14], phase separation [15], microemulsion [16], and electrochemical deposition [16].

Biological techniques involve the interaction of nanofiber raw materials with the enzymes and bacteria, some in the presence of external forces like mechanical pressure and temperature. Common example include when cellulose is treated with cellulase which cleave the fibers into simpler structures [17].

Non electrospinning techniques include centrifugal force or compressed gases instead of an electric field to generate nanofibers. These techniques limit the use of solvent, increase the production, and lower the production cost. Common techniques include centrifugal spinning [18], gas-jet spinning [19], melt blown technique [20], and fiber drawing [21]. A high output centrifugal spinning is not recommended for the biomedical application due the erratic behavior of the nanofibers which might affect the bioactivity of the fibers [22].

1.4 Electrospinning technique for nanofiber production

Electrospinning is among the most widely employed technique in the 21st century for its ability to fabricate various structure adaptable to different applications. Nanofibers developed by electrospinning technique have found its applications in the fields of nanoelectronics, optics, protective clothing, acoustics applications, filtration membranes, and many biomedical applications [23]. The ability of the electrospinning process to control the fiber fineness, surface morphology, orientation and cross-sectional configuration, and its ability to process a wide variety of material make it the most suitable over the other rival spinning technologies like: melt blown, centrifugal spinning, bicomponent spinning, phase separation, fiber drawing, and microemulsion etc.

1.4.1 Fundamental of electrospinning process

Electrospinning is a simple and inexpensive spinning process which uses electric field to transform the polymer from solution or melt to elongate into nano/micro fibrous structure. A schematic of electrospinning is shown in Fig. 1.1. Briefly, the polymer solution/melt is placed in a syringe attached to a needle. The syringed is placed in an external pump which ejects the solution at the needle tip. The needle tip is attached to a high voltage power supply, where a high electric voltage/potential is applied. The needle is placed in front of a collector usually having zero potential. As the applied potential increase at the needle tip, the electric force overcomes the surface tension of the polymer solution /melt. At this instance a polymer jet is formed at the needle tip and the droplet at the needle tip elongates to a Taylor cone, from where the polymeric nanofibers originate and then deposit on the collector. The elongation of the polymeric nanofibers results in the evaporation of the solvent and the nanofiber mat is deposited on a grounded collector.

Today the electrospinning equipment are rapidly moving to commercialization. Bubble electrospinning, melt electrospinning, coaxial electrospinning, and nano spider electrospinning are a few common examples.

In electrospinning the nanofiber diameter, morphology, alignment, as well as molecular orientation are affected by the nature of the collector, applied voltage, distance between nozzle and collector, and dispersion flow rate [24].

Among several nanofiber processing methods, co-electrospinning, side by side electrospinning, multi jet electrospinning, coaxial electrospinning, emulsion electrospinning are recognized as the easiest and effective methods for drug delivery and biomedical application [25].

Nanofiber morphology in electrospinning techniques is governed by the following parameters: (1) electrospinning parameters, for example, applied electric field, distance between needle and collector, flow rate and needle diameter, (2) solution parameters for example, type of solvent, polymer concentration, viscosity of solution, and conductivity of the solution, (3) environmental factors i.e., relative humidity and temperature [26].

Figure 1-1 An illustration of electrospinning setup.

1.5 Literature review

Electrospun cellulose acetate are not completely biodegradable within the human body due to the lack of cellulose enzyme in the human body. That is why it has found its application as topical drug delivery vehicle. Acetyl groups per cellulose acetate monomer plays the vital role in determining solubility, surface hydrophilicity, and thermal behavior. The mechanical performance of the cellulose acetate depends upon the inter chain crosslinks and can be altered by the addition of another polymer or agent. Drugs and bioactive agents are encapsulated in cellulose acetate

nanofibers for controlled and targeted delivery. They were employed in transdermal or topical delivery of drugs systems, wound care, and other biomedical applications [27].

The use of natural products is becoming a popular trend in both medicinal and food preservation applications. The increase in their popularity is due to their low toxicity [28]. The current problem of antibiotic resistance has prompted researcher to revert to the use of natural medicinal products like honey and essential oils from plants [29].

Honey, a natural material formed by bees has been highly regarded for its medicinal properties. The advent of antibiotics in the 1900s dejected the use of natural products for wound care. However, antibiotic-resistant microorganisms have encouraged researchers to revert back to natural products [30]. The antibacterial activity of honey is attributed to its multiple ingredients, enzymatic production of hydro- gen peroxide H_2O_2, non-peroxidase methylglyoxal (MGO), antimicrobial peptide bee defensin-1, and other compounds such as polyphenols that have not been entirely explained [31]. Besides, honey possesses low pH and high osmolality which hinders bacterial growth. H_2O_2 in honey is produced by glucose oxidase, which converts glucose into H_2O_2 and gluconic acid. Honey contains H_2O_2 in a very low amount but still has a disinfectant without being cytotoxic [32]. Kwakman et al. [33], reported that no H_2O_2 was detectable in Manuka honey antimicrobial activity thus suggesting that non-peroxidase factors are responsible for Manuka honey antibacterial activity. MH is prepared by bees from the nectar of Leptospermum scoparium flowers from New Zealand. MGO is the active antibacterial agent present naturally in MH. MGO is produced by non-enzymatic conversion of dihydroxyacetone present in nectar of the Leptospermum scoparium tree [34]. MGO is not the only factor for the antibacterial activity of MH. Other components like poly- phenols are also responsible for MH activity. Plants nectar contains polyphenols characterized by multiple phenol units. These polyphenols are also antioxidant i.e. flavonoids. The flavonoid's antibacterial activity is associated with their inhibition of bacterial energy metabolism, cytoplasmic membrane function and DNA gyrase [28]. Honey application on wounds promotes a moist environment which accelerates healing and guarantees re-epithelialization and non-adherence of the applied dressing to the wound surface, thus protecting the newly formed tissues [35].

Blumea balsamifera (BB) is an important medicinal plant from the genus Blumea. It is also known as “Sambong” and is being used as an herbal medicine for thousands of years in Southeast Asian

countries like Malaysia, Vietnam, China, and Thailand [36], [37]. The essential oil ex- tract from the leaves and stems of the plant is used to treat dermatitis, lumbago, beriberi, eczema, wounds, and also as an insecticide [38]. The extract of the plant has reported possessing antioxidant, antifungal, antibacterial, and antitumor properties [39]. The leave extracts of BB contain considerable amounts of flavonoids and volatile oils [40]. Volatile oil analysis by GC–MS revealed that the L-borneol (33.2%), caryophyllene (8.24%), Iedol (7.12%), and D-camphor (17.76%) are the most abundant components [41]. L-borneol is an essential ingredient used in healing burn wounds by Chinese herbal medicine practitioners. It has reported fastening wound healing by stimulating the expression of cytokines and growth factors and influencing the anti-inflammatory responses [42]. Pang et al. reported that using BB oil externally on the wound model in mice promoted capillary maturation, collagen de- position, granular tissue formation, and wound contraction [43]. Wang et al. reported BB oil was not allergic to the intact skin of the guinea pigs and had no acute toxicity to the rats in both intact and damaged skin models [44].

Biologically active agent may simply be amalgamated with polymer solution, forming core/shell structure, and physically or chemically surface immobilized or entrapped into fibers. Therapeutic agents loaded in nanofibers have been in use for transdermal drug delivery, wound care, and post-surgery tissue engineering applications [45]. CA fibers have been incorporated with various therapeutic agent ranging from antibacterial, antioxidant, anti-inflammatory, and antiviral agent. Studies have shown that a broad array of therapeutic agents can be loaded into nanofibers [46]. Kamyar et al. reported propolis impregnated CA/PCL nanofibrous mats for antibacterial and antioxidant applications. The propolis CA/PCL nanofiber showed high antioxidant activity and were effective against both Gram negative and Gram-positive bacteria [47]. Sustained release CA and ethyl cellulose blend nanofibers loaded with ketoprofen were developed by Um-i-Zahra et al. [48], they reported that using a blend system can help in fabricating a system which can physically and chemically entrap drugs and make it possible to fabricate other novel drug releasing systems. Pattama et al. developed vitamin loaded CA nanofiber mats for transdermal and dermal delivery systems. They reported that drug loading efficiency of the vitamin was dependent on the formulation of the sol- vent used for electrospinning and furthermore, they established that drug release from the nanofibers was sustained as compared to the as cast films of CA [49].

Patrojanasophon et al. [50] reported the release of water-soluble drug from CA nanofibers depending upon the degree of alignment of the nanofibers on the collecting mandrel. They found

out that randomly oriented nanofibers showed controlled release due to formation of an entangled mesh network affecting the diffusion of drug to the external medium. While highly oriented nanofiber showed an abrupt release. So, by manipulating the orientation of nanofibers one can design and abrupt or controlled release CA nanofiber-based drug delivery system. Wutticharoenmongkol et al. [51] fabricated Gallic acid loaded CA nano- fibers with 10–40% by weight of Gallic acid. The nanofibers showed antibacterial efficacy against S. aureus and retained it antioxidant activity in both as spun and as released condition, implying that the gallic acid loaded in the CA did not lose it bioactivity. Liu et al. [52] prepared rectorite and paclitaxel immobilized cellulose acetate nanofibers. They reported that the CA served as an effective polymer for loading of rectorite and paclitaxel. Furthermore, the addition of rectorite improved the immobilization of paclitaxel in the CA nanofibers and have synergistic cytotoxicity effect on the gastric cancer cells (SGC7901). Hua et al. [53] fabricated the pH responsive polyurethane/core and cellulose acetate phthalate/shell nanofiber for intervaginal drug delivery. These core shell fibers showed improved mechanical strength as compared to the neat cellulose acetate phthalate. The cellulose acetate phthalate imparts pH responsiveness to the core-shell structure giving the fibers potential for "semen sensitive" (intravaginal) drug delivery. Pereira et al. developed chitosan nano whiskers coated CA electrospun nanofibers. The coated nanofibers were effective against gram negative bacteria and were a toxic to healthy Vero cells [54].

Liakos et al. reported the CA-lemongrass essential oil nanocapsule. The nanocapsules adhered to the mucous membrane well and were effective against Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) [55]. Vongsetskul et al. report CA nanofibers loaded with A. ebracteatus Vahl. ~99% of the loaded extract was released by diffusion in acetate buffer solution and was active with antioxidant activity along with moderate toxicity [56]. Liakos et al. reported the rosemary and oregano oil loaded CA nanofibers. They stated that the CA electrospun pads with 5% essential oil were effective against bacterial and fungal strains [57]. In another work, Liakos et al. reported grafting of pepper- mint, cinnamon, and lemongrass on the CA chain and produced antibacterial and antifungal nanocapsules [58]. Fawal et al. reported rosemary and Aloe vera essential oils loaded CA films. They reported the effectiveness of the films against E. coli and Bacillus subtilis (B. subtilis). The inclusion of essential oils also improved the antioxidant activity of the CA films [59].

A variety of synthetic and natural fillers are available as reinforcement agents to improve the mechanical properties and bioactivity of the polymeric scaffolds. Clay has emerged as one of the inorganic fillers that can substantially improve the mechanical properties and bioactivity and have been previously studied in processing bone cement [60]–[63]. Illite is a type of non-expanding clay found in temperate and cold regions. It has a dioctahedral 2:1 structure having one octahedral layer sandwiched between two tetrahedral sheets [64]. Illite possesses a large specific surface area and can serve as a promising adsorbent. Most of the previous studies focus on the illite usage as a heavy metal ion adsorbent from the solution [65]. Illite can also adsorb organic moieties such as tetracycline because of its cation exchange capacity and large specific surface area [66]. Based on these properties of Illite, we are hypothesizing that illite addition will improve the hydroxyapatite mineralization over the scaffold. Illite has a long history as a therapeutic agent for various diseases involving cerebrovascular, phlegm dispersing, and activation of blood circulation and Qi-tonifying, according to Dongui Bogam, the Korean medicinal book. In 2015 Jeon et al. reported that Illite could be used to treat Alzheimer's disease [67].

Zein is a corn protein encompassing ~80% of the protein present in the corn grain. It is a "green" and renewable substitute for petroleum-based or animal-derived polymers in biomedicals. Due to its plant origin, it is rendered non-immunogenic compared to the other protein polymers obtained from animal sources [68]. Among all the plant-based proteins, Zein, a corn protein rich in glutamine, leucine, alanine, and proline, has shown potential as a biomaterial for bone regeneration. Zein improves cell adhesion by interacting with the tissue transglutaminase, an extracellular enzyme [69]. However, Zein offers many advantages, including biocompatibility, biodegradation, adequate strength, low immunogenicity; however, it lacks bioactivity [70].

Many polyesters have been studied for tissue engineering applications in the past decade due to their biocompatibility, bioresorbable, and high mechanical strength [71]. Among them, Polycaprolactone (PCL) is of great interest in tissue engineering. PCL is an aliphatic polyester with a low glass transition temperature (-62°C) and a melting point ranging between (55—60°C). Apart from being biocompatible and biodegradable, PCL qualifies for tissue engineering applications due to its ease of availability, low cost, ease of processing, and modification. PCL's biological and mechanical characteristics make it an excellent candidate for musculoskeletal tissue engineering applications [72]. However, some of the drawbacks of the PCL include its slow in vivo degradation

and lack of bioactive nature, including hydrophobicity which hinders cell proliferation and adhesion [73].

To over these drawbacks of PCL, blending PCL with a suitable natural organic/inorganic bioactive component is a convenient way to impart some hydrophilicity and improve degradation rate without sacrificing the biocompatible nature of the polymer. For example, acidic degradation byproducts of PCL and the degradation rate of PCL were both enhanced by blending with chitosan [74]. Blending PCL with polysaccharides like starch, dextran, and gellan can improve the cell adhesion and proliferation of the composite scaffolds [75]. Wu et al. prepared Zein/PCL blend fibrous scaffold and reported improvement in hydrophilicity and degradation rate was certainly improved [76]. Dong et al. reported excellent toughness and flexibility of PCL/zein films[77]. Liao et al. developed Zein/PCL from two nozzle electrospinning setups. They said that adding calcium lactate to the composite scaffold improves in vitro mineralization, cell adhesion, spread, and proliferation [78]. He et al. developed coaxial core/shell PCL/Zein nanofibers. They achieved the controlled release of metronidazole and reduced toxicity because of the controlled release [79]. Awasthi et al. fabricate albumin exfoliated MoS2 incorporated PCL/Zein nanofibrous scaffolds. They reported increased tensile properties, cell adhesion, and osteoblastic differentiation. In contrast, the degradation rate was slowed done due to the incorporation of MoS_2 [73].

1.6 Objectives

The study focuses on the development of naturally derived bioactive agents loaded polymeric nanofibers using electrospinning technique. The target was to develop bioactive nanofibers for regenerative application particularly wound healing and bone tissue engineering.

The objectives are briefly listed below:

1) Successfully fabricate bioactive agent loaded nanofibrous mats by electrospinning.
2) To evaluate the produced nanofibrous mats for their physicochemical properties.
3) The perform detailed in vitro analysis for the biological characterization of the produced nanofibrous mats.

Highlights of the research outcomes chapter wise are given below.

Chapter 2

- Bead-free CA-MH electrospun nanofibrous mats were successfully fabricated.
- Nanofiber mats showed high porosity suitable for wound breathability.
- CA-MH mats favor cell growth and proliferation.
- Nanofibrous mats were efficient against E. coli and S. aureus.
- CA-MH mats showed good overall in vitro performance as potential wound dressing.

Chapter 3

- Ultrafine bead-free BB oil loaded CA fibrous mats were prepared by electrospinning.
- FTIR spectra indicated the retention of BB oil in CA mats after electrospinning.
- The mats showed good antibacterial, cell viability, and antioxidant properties.
- A biphasic release was observed and followed the first-order release kinetics.
- MVTR values 2450 – 1750 g/m2/day are adequate for the wound breathability.

Chapter 4

- Illite clay was successfully incorporated into PCL/Zein electrospun nanofibers.
- Physicochemical properties were tunable by varying the amount of Illite clay.
- Illite addition enhanced the biomineralization ability of the composite scaffolds.
- CCC scaffolds showed improved osteoblast differentiation confirmed by ALP staining.
- Illite clay enhanced the biocompatibility for MC3T3-e1 cells in in vitro assessment.

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Chapter 2

Manuka Honey Incorporated Cellulose Acetate Nanofibrous Mats: Fabrication and *In Vitro* Evaluation as a Potential Wound Dressing.

2.1 Introduction

Human skin serves as the first line of defensive barrier against environmental factors such as pathogens. When the skin is harmed, it should be secured by the appropriate dressing depending on the intensity of the wound. Otherwise, bacterial growth from the human skin flora can result in infection and impede the healing process [1]. In particular, the diseases caused by multi-drug-resistant bacteria pose an increasing threat to human health [2]. Consequently, finding an alternative for treatment have encouraged scientist to revert to natural medicinal sources and evaluate their wound healing efficacy [3].

Honey, a natural material formed by bees, has been highly regarded for its medicinal properties. The advent of antibiotics in the 1900s discouraged the use of natural products for wound care. However, antibiotic-resistant microorganisms have encouraged researchers to revert to natural products [4]. The antibacterial activity of honey is attributed to its multiple ingredients, enzymatic production of hydrogen peroxide H_2O_2, non-peroxidase methylglyoxal (MGO), antimicrobial peptide bee defensin-1, and other compounds such as polyphenols that have not been entirely explained [5]. Besides, honey possesses low pH and high osmolality, which hinders bacterial growth. H_2O_2 in honey is produced by glucose oxidase, which converts glucose into H_2O_2 and gluconic acid. Honey contains H_2O_2 in a meager amount but still has a disinfectant without being cytotoxic [6]. Kwakman et al. [7] reported that no H_2O_2 was detectable in Manuka honey antimicrobial activity, thus suggesting that non-peroxidase factors are responsible for Manuka honey antibacterial activity. MH is prepared by bees from the nectar of Leptospermum Scoparium flowers from New Zealand. MGO is the active antibacterial agent present naturally in MH. MGO is produced by non-enzymatic conversion of dihydroxyacetone in the Leptospermum Scoparium tree [8]. MGO is not the only factor for the antibacterial activity of MH. Other components like polyphenols are also responsible for MH activity. Plants nectar contain polyphenols characterized by multiple phenol units. These polyphenols are also antioxidants, i.e., flavonoids. The flavonoid's antibacterial activity is associated with inhibiting bacterial energy metabolism, cytoplasmic membrane function, and DNA gyrase [9]. Honey application on wounds promotes a moist environment which accelerates healing and guarantees re-epithelialization and non-adherence of the applied dressing to the wound surface, thus protecting the newly formed tissues [10].

In a study by Santos et al. [5], cryogels of poly (vinyl alcohol) with sodium carboxymethylcellulose, gelatin, and starch infused with MH that composition superior to 25% MH content were

bacteriostatic, indicating a need for a higher quantity of MH to be more effective. Another study reports that green electrospun MH/silk fibroin nanofibrous matrices were implanted on a 9mm wound in a mice model. The wound size significantly decreased over 12 days due to the incorporation of honey [11]. Furthermore, the MH inclusion in poly ε-caprolactone (PCL) resulted in increased osmotic pressure. Increased proliferation and migration and bacterial clearance of S. aureus and E. coli were reported [12].

Electrospun scaffolds have offered a unique avenue for MH delivery while providing a matrix for wound regeneration due to their flat geometry. Nanofiber fabrication is one of the most prominent features of nanotechnology. A high aspect ratio (surface to volume) of nanofibers can result in high drug incorporation and mass transportation [13–15]. The most sustainable natural polymeric material, cellulose, has found remarkable consumption as electrospun nanofibers. Cellulose is largely considered for biodegradability, biocompatibility, robust availability as a renewable source, and economic viability. Due to its limited solubility in general organic solvents and its disability to melt due to excessive inter and intra-molecular hydrogen bonds, Cellulose hinders its biomedical application [16]. To overcome this issue of solubility, various derivatives of cellulose are investigated. Of these studied derivatives, cellulose acetate (CA) has been extensively employed for the fabrication of electrospun nanofibers. CA found its application biomedical field due to its outstanding properties of biodegradability, biocompatibility, hydrolytic stability, good mechanical strength, insolubility in water, non-toxicity, ready availability, and low cost [17]. CA can be potentially used for wound care and primarily as antimicrobial membranes, tissue scaffolds, bio nanocomposites, and biomedical separators [16,18–20].

The biologically active agent may be amalgamated with the polymer solution, forming core/shell structure, and physically or chemically surface-immobilized or entrapped into fibers. Therapeutic agents loaded in nanofibers have been used for transdermal drug delivery, wound care, and post-surgery tissue engineering applications [21]. CA fibers have been incorporated with various therapeutic agents ranging from antibacterial, antioxidant, anti-inflammatory, and antiviral agents. Studies have shown that a broad array of therapeutic agents can be loaded into nanofibers [22]. Kamyar et al. reported propolis impregnated CA/PCL nanofibrous mats for antibacterial and antioxidant applications. The propolis CA/PCL nanofiber showed high antioxidant activity and effectiveness against both gram-negative and gram-positive bacteria [23]. Phiriyawirut et al. reported gallic acid-loaded CA electrospun nanofibers. The fibers containing 2.5% gallic acid were

smooth surfaces but increasing the gallic acid content resulted in flake formation on the fiber surfaces. The release behavior of the gallic acid-loaded CA fibers was compared to as-cast film and was found to be much lower, thus resulting in controlled release [24]. Sustained-release CA and ethyl cellulose blend nanofibers loaded with ketoprofen were developed by Um-i-Zahra et al. [25]. They reported that using a blended system can help fabricate a system that can physically and chemically entrap drugs and make it possible to fabricate other novel drug releasing systems. Pattama et al. developed vitamin-loaded CA nanofiber mats for transdermal and dermal delivery systems. They reported that the drug loading efficiency of the vitamin was dependent on the formulation of the solvent used for electrospinning, and furthermore, they established that drug release from the nanofibers was sustained as compared to the as-cast films of CA [26].

In this present work, we fabricated MH-loaded CA nanofibrous mats by electrospinning. The surface morphology, secondary structure, surface area, porosity, physicochemical, antibacterial activity and in vitro cytocompatibility of the resultant nanofibrous mats were studied.

2.2 Experimental

2.2.1 Materials

Cellulose acetate (~40%) weight content, Mw 30000, was procured from Sigma Aldrich (USA). Manuka Honey MGO 550+ was purchased from Manuka Health New Zealand Ltd. Acetone (99.5%), and *N-N* Dimethylformamide (DMF 99.8%) were obtained from Wako Chemicals Ltd (Japan).

2.2.2 Preparation of electrospun cellulose acetate – manuka honey (CA-MH) nanofibers

Cellulose acetate spinning solution was prepared by dissolving 20% by weight CA in DMF/Acetone, 3:2, solution. The spinning solution was agitated for 24 hours on a magnetic stirrer at 1200 rpm to ensure homogeneity. A horizontal arrangement of high voltage power supply (Har-100*12, Matsusada Co, Tokyo, Japan), a rotating cylindrical collector, and a syringe pump (KDS-100, KD Scientific, USA) was set up for electrospinning. The spinning solution was filled in a 20ml plastic syringe fixed with a 20-gauge needle (0.9mm diameter). A high voltage of 15kV was applied at the needle tip. The flow rate was kept at 0.7 ml/h. The distance between the needle tip and the collector was maintained at 200mm. The nanofibrous mat was collected on butter paper wrapped over the collector. The electrospinning was carried out at an ambient temperature of 25°C and relative humidity of 55%. For making composite CA/MH nanofibers, MH was added to the

above formulation in 10%, 15%, and 20% by weight (with respect to CA). The nanofibers produced were labeled as CA, CAH1, CAH2, and CAH3. The processing parameters for the blend CA/MH nanofibers were the same as that of the neat CA.

2.2.3 Characterization

2.2.3.1 Surface morphology

The surface morphology and nanofibers diameter were studied by scanning electron microscopy (SEM, JSM-6010LA, JEOL, Japan). The nanofiber samples were sputter-coated with platinum before SEM imagery. 50 nanofibers were randomly selected from the SEM images, and their averages were recorded as mean nanofiber diameters. SEM micrographs were analyzed using ImageJ (version 1.4.3) software.

2.2.3.2 Physicochemical analysis

The physicochemical analysis of the produced nanofiber mats was carried out by employing Fourier-transform infrared spectroscopy (FTIR) with a Prestige-21, Shimadzu Co. Ltd., Japan, in Attenuated total reflectance (ATR) mode, with a range of 4000-600 cm-1 wavenumber.

2.2.3.3 XRD analysis

The X-ray diffractograms of the neat and honey-loaded nanofiber mats were determined using a nickel-filtered Cu-Ka radiation-assisted XRD diffractometer (Rotaflex RT300 mA, Raigaku Corporation, Osaka, Japan). The scanning speed was kept at 5° per minute, and the 2θ range was between 5° and 80°.

2.2.3.4 Tensile test

To study the mechanical behavior of the produced nanofibers tensile test was carried out using the universal tensile testing machine (UTM), Tensilon RTC 250A, A&D Company Ltd. Japan. Nanofiber mats were cut into small rectangular samples with uniform length (40mm) and width (3mm), but the thickness of the samples was not the same. The gauge length of the samples was constant at 20mm, with the rate of traverse kept at 5mm/min. Stress, strain, and young's modulus of the nanofiber mats were obtained from UTM data using equations 1, 2, and 3 given below:

$$Stress\ (\sigma) = \frac{F}{A} \quad (1)$$

$$Strain\ (\varepsilon) = \frac{\Delta l}{l} \quad (2)$$

$$Modulus\ (E) = \frac{Stress\ \sigma}{Strain\ \varepsilon} \quad (3)$$

2.2.3.5 Surface area and pore size distribution

The Brunauer-Emmett-Teller (BET) specific surface area (SSA) and pore size distribution of electrospun nanofiber mat of CA and MH loaded CA nanofiber mats were analyzed using nitrogen (N_2) physisorption employing SHIMADZU Tri-Star II-3020, Japan. The specimen weight was fixed to 500mg. Each specimen was degassed at 80°C for 1 hour in N_2. Total pore volumes were measured by the Barret-Joyner-Halenda (BJH) adsorption method.

The nanofiber mats were cut into 4cm × 4cm squares and subsequently weighed to measure porosity. The thickness of the mats was measured using a digital multimeter at five different places and averaged out for further calculations. The apparent volume (V_a) was measured using the dimensions of the previously cut nanofiber mat. The volume (V_g) was determined by the density of the manuka honey (1.42 gcm^{-3}) and cellulose acetate (1.3 gcm^{-3}) and their weight ratio in the respective specimen. Porosity was measured using the following equation 4 [27–29]:

$$Porosity\ \% = \left(1 - \frac{Vg}{Va}\right) \times 100 \quad (4)$$

2.2.3.6 Water vapor transport rate (WVTR)

To evaluate the WVTR of the electrospun nanofiber mats by ASTM E96-95, the nanofiber mats were cut in a circle with 9 cm diameters and placed over the mouth of cups with 7 cm diameters. The cups were filled to three-quarters level with distilled water and weighed "W1". The cups were placed in an incubator under 40% humidity at 37°C. The cups were removed from the incubator after 24 hours and weighed again "W2". WVTR was calculated from the following equation 5;

$$WVTR = G/At \quad (5)$$

Where "G" is the change in weight (W1 –W2), "A" is the area of the cup mouth, and "t" is the time in which "G" happened.

2.2.3.7 Water contact angle

The wetting behavior of the composite CA-MH nanofiber mats was determined by water contact angle using contact angle analyzer Digidrop, GBX, Whitestone Way, France. A static water contact

angle was measured using a 28-gauge needle. Five readings for each type of fiber mat were measured, and means were calculated.

2.2.3.8 Antibacterial evaluation of CA-MH nanofiber mats

The qualitative antibacterial activity of the electrospun CA-MH nanofiber mats was determined by the agar plate disc diffusion method (AATCC 147-1998) as reported earlier [30]. A 50µL aliquot of 10^{-5} dilution of overnight prepared gram-negative Escherichia Coli and gram-positive Staphylococcus Aureus was spread over agar plates. Nanofibrous mats were cut in circular discs with 10mm diameter. The sample discs were pressed gently against the agar surface to ensure intimate contact. The agar plates were incubated for 24 h at 37ºC. Afterward, these plates were examined for interruption of bacterial growth, both underneath and for the zone of inhibition along the circumference of the nanofiber discs.

The optical density (OD) technique was used to measure the PAC fibers' antimicrobial activity quantitatively. The test was conducted as previously reported [31]. Briefly, a single colony from the culture collection of *E. coli* and *S. aureus* was inoculated in 100ml of nutrient broth and incubated at 37ºC for 24 hours under stirring. A 10^{-4} dilution of the cultures was prepared. A 10 µl of aliquot was dispensed in bottles containing 9.5ml nutrient broth solution. Fiber samples (200mg each) were added to the respective bottles and incubated for 24 hours. Afterward, the absorbance of the blank and fiber sample containing broth solutions was measured at 600nm using a UV-Vis spectrophotometer (Lambda 900, Perkin-Elmer, USA). The blank was prepared in the same way but did not hold any sample. Readings were taken in triplicate, and averages were taken. The bacterial reduction was calculated using equation 6;

$$Bacterial\ Reduction\ \% = \frac{Abs\ blank - Abs\ sample}{Abs\ blank} \times 100 \quad (6)$$

Where "Abs_{blank}" is the absorbance of the blank bottle and "Abs_{sample}" is the absorbance of bottles containing fiber samples.

2.2.3.9 Cell viability analysis (WST-1 assay)

The in vitro cytocompatibility of the electrospun CA-MH nanofiber mats was analyzed by WST-1 assay. WST-1 assay determines the cleavage of tetrazolium salt to formazan by cellular mitochondrial dehydrogenases. So, the higher the viable cell in the medium, the more will formazan, resulting in higher chromatic absorption, thus higher cell viability. NIH 3T3 mouse fibroblast cells

were cultured in Dulbecco modified eagle medium (DMEM) accompanying 10% fetal bovine serum (FBS) using a humidified incubator at 37ºC having a 5 % CO_2 atmosphere. The nanofiber mat specimen (3 mm discs) were sterilized with 70 % ethanol for 10 minutes and later washed with phosphate buffer saline solution (PBS). The sterilized specimen were placed in 96 well glass culture plates. The mat-free wells were used as controls. The wells were seeded at a density of 1×10^3 NIH 3T3 cells per well. The seeded wells were cultured for 1, 3, 5, and 7 days. At the point of 1, 3, 5, and 7 days, 10 µL WST-1 was added to each well and incubated for two hours. Absorbance was measured at 440 nm using a microplate reader (Thermo Scientific, Multiskan FC Instrument). Readings were measured in triplicate, and averages were recorded.

To study the NIH 3T3 fibroblast cell line interaction with the nanofibrous mats, the morphology of NIH 3T3 on CA-MH mats was observed with SEM photographs. After three days of culture, cells were fixed on the mats with 4% paraformaldehyde for 4 hours at 4ºC. The nanofibrous mats were dehydrated using 10, 30, 50, 70, and 100 (v/v) ethanol. The samples were vacuum dried before taking SEM photographs.

2.2.3.10 Entrapment efficiency and release behavior

Intended drug loading can serve as an important tool to determine the overall entrapment efficiency (EF) of the nanofiber mats. To determine the encapsulation efficiency, 100 mg of MH-loaded nanofiber mats were dissolved in acetone. These solutions were examined against the standard fitted line plot (Figure 14 b) of manuka honey using an ultraviolet-visible (UV-Vis) spectrophotometer (Lambda 900, Perkin-Elmer, USA), and adjusting it with 9.8 from the standard calibration curve (1-10 mg/ml); Y= 9.837X + 0.3798, R^2= 99.6%. By doing this, we were able to calculate the actual honey content in the nanofiber mats. Loading efficiency was measured by using the following equation 7 [32].

$$EF\ (wt\ \%) = \frac{Actual\ honey\ content\ in\ nanofiber\ mats}{Theoratical\ honey\ content\ in\ nanofiber\ mats} \times 100 \quad (7)$$

Release behavior of MH loaded CA nanofiber mats were measured by dipping 300 mg of nanofiber mats in 20 ml of phosphate buffer saline solution PBS (pH 7.2 - 7.4) in glass bottles, which were kept in an incubator at 37 ºC. At predetermined time intervals between 0 to 48 h, 1 ml of solution was extracted, and honey release was measured. Simultaneously, an equal amount of PBS was supplemented to keep the sink settings constant. The maximal spectral absorbance of MH was predetermined at wavelength 275 nm (λ_{max}) using UV-VIS spectrophotometer (Lambda 900,

Perkin-Elmer, USA). The absorbance spectra of the MH were converted to concentration by using a calibration curve of MH in PBS. Release behavior was calculated as % cumulative release (equation 8) with respect to time (h) [33–35].

$$Release\ (\%) = \frac{Ct}{Ctotal} \times 100 \quad (8)$$

Here, "C_t" is the release concentration at time "t" and "C_{total}" is the total drug release after the nanofiber mats were dissolved. The release was measured in triplicate for each specimen, and results were presented as averages.

2.2.3.11 Antioxidant assay

Free radical scavenging activity is determined using 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH). DPPH when reacted with a free radical scavenger it reduces and changes its color from purple to yellow based on the reducing efficacy of the antioxidants. To analyze the antioxidant effectiveness of the CA-MH nanofibrous mats, 50 mg of each sample was soaked in 3 ml of 0.1mM DPPH methanol solution and incubated for different time periods (2, 6, and 12 h) at 37°C in an incubator under dark conditions. DPPH solution without sample was considered as a control. At the set time intervals, the absorbance of the solution was monitored at 517 nm using a UV-VIS spectrophotometer (Lambda 900, Perkin-Elmer, USA). The antioxidant activity was measured in percentage using the following equation 9.

$$Antioxidant\ efficiency\ (\%) = \frac{A\ control - A\ smaple}{A\ control} \times 100 \quad (9)$$

Where $A_{control}$ and A_{sample} is the absorbance of DPPH solutions without and with nanofiber mats at 517 nm.

2.3 Results and Discussion

2.3.1 Morphology of nanofibrous mats

The neat CA nanofiber from 20% by weight polymer solution and MH loaded composite nanofibers with different quantities (10%, 15%, and 20% (w/w) to CA quantity) were successfully electrospun. The actual photographs of the samples are shown in Fig. 2.1.

Figure 2-1 Actual images of the original samples (a) CA, (b) CAH1, (c) CAH2, and (d) CAH3.

When the quantity of the MH was increased further than 20% (w/w), it wasn't easy to produce uniform nanofibers without beads (Fig. 2.2).

Figure 2-2 SEM photograph of CA with 25% weight content of MH.

The SEM micrographs along with nanofiber diameter distributions are shown in Fig. 2.3. The micrographs Fig. 2.3 (a), (b), (c), and (d) illustrate that the nanofibers produced are continuous and bead-free. The mean nanofiber diameter for neat CA (n = 50) is 270 nm (Fig. 2.3 a'). MH loaded composite nanofibers diameter increased from 357 nm to 389 nm and 403 nm as the quantity of the MH in the composite fibers increased from 10% to 20% (Figure 2.3 b', c', and d'). The increase in diameter is assumed as a resultant of the successful incorporation of MH in the base CA nanofiber.

Figure 2-3 SEM micrographs and diameter distribution histograms of the (a, a') CA nanofibrous mat, (b, b') CAH1 nanofibrous mat, (c, c') CAH2 nanofibrous mats, and (d, d') CAH3 nanofibrous mats.

2.3.2 Physicochemical analysis

ATR fingerprints of CA are shown in Fig. 2.4. A weak broad peak can be observed at 3452 cm^{-1} due to OH stretching. A twin peak at 2963 cm^{-1} and 2902 cm^{-1} are an indication of CH_2 groups stretching. A peculiar ester groups COO^{-1} stretching are observed at 1739 cm^{-1}. At 1371 cm^{-1} methyl groups CH_3 stretching's can be observed. Strong peaks are 1226 cm^{-1} and 1031 cm^{-1} are due to C—O, and C—O—C of the acetyl and pyranose ring. C1—H twisting is observed at 908 cm^{-1}. The peaks are in accordance with the cellulose acetate and the literature [36–38] .

Figure 2-4 Comparison of the FTIR fingerprints of neat CA, CAH1, CAH2, CAH3 and MH.

ATR spectrum of Manuka Honey Fig. 2.4 shows a strong peak at 3321 cm^{-1} which is related to free water O—H and the primary amide N—H of proteins in honey. A double peak at 2930 cm^{-1} and 2875 cm-1 represents the CH_2 stretching. As methylglyoxal (MGO) is a major component in manuka honey, which is a ketoaldehyde showing a peculiar peak at 1645 cm^{-1} attributed to keto carbonyl group C═O, another peak at 1340 cm^{-1} can be assigned to CH_3 of the MGO [39]. The small peaks at 867 cm^{-1}, 819 cm^{-1} and 779 cm^{-1} can be attributed to monosaccharide and disaccharide sugars in the honey [40]. C—H bending of the carbohydrate is shown by the peak at

918 cm^{-1}. C—N vibrational band can be observed at 1421 cm^{-1}. The peaks at 1253 cm^{-1} and 1026 cm^{-1} are due to C—O stretching of C—OH group and C—C–H deformation in the carbohydrate backbone [11,41,42].

A few differences can be observed in the ATR spectrum Fig. 2.4 of the MH loaded CA nanofiber mats. The shifting of the weak and broad peak from 3452 cm^{-1} to 3360 cm^{-1} and the presence of the peak at 1654 cm^{-1} (keto carbonyl C═O) in the spectrum of the MH loaded CA nanofibers, confirm the successful inclusion of MH in the composite fibers.

2.3.3 XRD analysis

Fig. 2.5 illustrates the XRD diffractograms of the CA, CAH1, CAH2, and CAH3. It is an established fact that addition of impurity in a polymer result in deterioration of crystalline structure as the incorporated impurity cause misalignment in the polymer chains [43]. CA diffraction pattern shows an amorphous microstructure with two broad peaks at 2θ = 10o and 19o. This diffraction pattern is in accordance with the previously reported literature [38]. The MH inclusion in the nanofiber mats decreases the intensity of the peaks in diffraction pattern of the CA nanofiber mats. This is an indicator of deterioration in the crystalline structure of polymer backbone [44].

2.3.4 Mechanical performance

As the nanofiber mats were developed for wound care application. It must provide adequate mechanical properties and flexibility for clinical handling and wound healing. Literature reports that the skim mechanical properties varies between 0.1 – 32 MPa for strength and 0.42 – 2.26 stretch ration at break [45]. As the skin structure possess heterogeneity these values are not absolute and are dependent on several factors like genetics, age and color of the skin [46]. The mechanical properties of neat and MH loaded CA nanofiber mats are illustrated in Fig. 2.6. The strength at break of the MH loaded nanofiber is lower than the strength of the neat CA nanofibers. The decrease in the strength can be attributed to the misalignment of the molecules in the CA nanofiber upon inclusion of manuka honey. The strength at break for neat CA nanofiber was 1.61 MPa and that of MH loaded nanofiber was 0.91 MPa for CAH1, 0.67 MPa for CAH2, and 0.37 MPa for CAH3. The loss in tensile strength can be due to distortion in the polymer chain packing of CA nanofibers mats due to addition of MH. This can be attributed that the MH in the CA nanofibers is mechanically entrapped rather than having any chemical interaction. This effect is also visible in

Figure 2-5 XRD diffractogram of neat CA and composite CA-MH nanofibrous mats.

Figure 2-6 (a) Stress-strain curve and (b) Young's modulus for the neat and composite CA-MH nanofibrous mats.

XRD patterns of the neat CA and composite CA-MH nanofibers mats and is discussed in previous section. Similarly, a decreasing trend was observed in the young modulus (6.76 MPa for CAH1, 7.07 MPa for CAH2, and 3.68 MPa for CAH3) and elongation at break (13.9 % for CAH1, 8.3%

for CAH2, and 9.9% for CAH3) of the MH loaded nanofibers as compared to neat CA nanofibers (8.26MPa, 19.5%).

2.3.5 Specific surface area and porosity

The N_2 adsorption and desorption were utilized to analyze the structural porosity of the electrospun nanofiber mats. Fig. 2.7 shows the BET isotherms of the composite nanofiber mats. The unsaturated character at a relative pressure (P/P_o) near 1 and the hysteresis loop are indicators to classify the isotherms as mixed type II and type IV [47]. The BJH total pore volume, BET specific surface area, and porosity of the electrospun CA nanofiber mats with and without Manuka honey are summarized in Table 2.1. BJH total pore volume of the neat CA nanofibers was found to be 0.137 cm^3/g, which decreased to 0.112 cm^3/g, 0.101 cm^3/g, and 0.086 cm^3/g for CAH1, CAH2, and CAH3 respectively. Similarly, BET SSA of neat CA nanofiber mat was found to be 6.8 m^2/g which reduced to 6.4 m^2/g, 5.8 m^2/g and 5.2 m^2/g with the inclusion of MH (10%, 15%, and 20%) with respect to CA. This decrease in the SSA and total pore volume can be attributed to the increase in the nanofiber diameter and beads formation due to encapsulation of MH in the base CA nanofibers. The same decreasing trend can be seen in the porosity of the electrospun nanofiber mats of neat CA and composite MH loaded nanofiber mats. But still, the values of porosity are on a high side which should facilitate good transport of gasses and vapors between the wound microenvironment and the ambient atmosphere. The small pore size and high porosity of nanofibrous mats can facilitate gaseous exchange and bacterial isolation during wound repair [48].

Table 2-1 BET surface area and porosity values of neat CA and MH loaded nanofibrous mats.

Sample Labels	Specific surface area (m^2/g)	Total pore volume (cm^3/g)	Porosity (%)
CA	6.8257	0.137	89.41
CAH1	6.4725	0.112	87.48
CAH2	5.8524	0.101	86.87
CAH3	5.2311	0.086	85.38

2.3.6 WVTR analysis

Water vapor transmission plays a vital role in wound healing as it controls the hydration of the wound area. It has been reported that for ideal wound healing the best range of WVTR is to be 2000-2500 g/m^2/day [1,49,50]. A higher WVTR will causes dehydrated wound surface which eventually results in scar formation. On the other hand a lower WVTR will cause maceration around the wound surface, over accumulation of wound exudate and thus results in infections [51]. The produced composite nanofibrous mats with different composition of CA and MH exhibit WVTR in the range of 2600 to 1950 g/m^2/day as shown in Fig. 2.8. It is evident that as the MH content in the nanofibrous mats increases the WVTR decreases. This decrease could be attributed to the decrease in the surface area and porosity of the composite CA-MH nanofibrous mats due to increase in the fiber diameters. The WVTR of the CA-MH mats are acceptable range as has been reported in literature.

Figure 2-7 N_2 adsorption-desorption isotherms (a, b, c, and d) for surface area analysis and BET plots / Langmuir fits (e, f, g, and h) of neat CA (a, e), CAH1 (b, f), CAH2 (c, g) and CAH3 (d, h) nanofibrous mats.

Figure 2-8 WVTR of the electrospun mats with different CA-MH compositions.

Figure 2-9 Static water contact angles for neat CA, CAH1, CAH2, and CAH3.

2.3.7 Water contact angle

A static water contact angle was measured to determine the wetting behavior of the neat CA and composite CA-MH nanofiber mats Fig. 2.9. The mean water contact angle for the neat CA nanofiber mat was measured as 82.4º, this is concurrent with the hydrophobic nature of the cellulose acetate. The addition of MH in the composite CA-MH nanofiber results in the decrease of the water contact angles to 79º for CAH1, 74º for CAH2, and 68º for CAH3. As the MH content in the nanofibers increases from 10% to 20% the water contact angle decreases, this may be due to the presence of water-loving functional groups (mainly – OH groups on the carbohydrate chains and primary amines – NH on the protein) in the composition of honey. This increased hydrophilicity of CA-MH nanofibrous mats might help in cell adhesion and migration.

2.3.8 Antibacterial evaluation of CA-MH nanofiber mats

Fig. 2.10 shows the antibacterial zone of inhibition test of the neat CA and MH loaded CA nanofiber mats against gram-negative (a) *Escherichia Coli* and gram-positive (b) *Staphylococcus Aureus* bacterial strains. These bacterial strains are the most common cause of soft tissue infections and subsequently hinder the wound healing process. Most of the clinically acquired infections are resultant to *Staphylococcus Aureus.* While *Escherichia Coli* is considered as a primary source for infection in the burn wounds [52,53].

Halos (areas without bacterial growth) can be seen around the nanofiber discs loaded with MH which reveals the antibacterial efficacy of the MH against the gram positive and gram-negative bacterial strains. Furthermore, as the quantity of the MH in the composite nanofiber increases from 10% to 20% the clear zones also increase. The inhibition zones are calculated in millimeters and are given in Table 2.2.

OD of the gram-positive *S. aureus* and gram-negative *E. coli* in nutrient broth was measured at 600 nm (Fig. 2.11) with and without CA-MH nanofiber mats. The percentage reduction in the bacterial growth was measured and is reported in Table 3. It is clear from the values in Table 2.3 that as the MH content in the nanofibrous mats increases the bacterial reduction increases. The statistical significance of these values was determined using one-way analysis of variance (ANOVA). The output of the ANOVA is given in Table 2.4. The ANOVA results justify that MH concentration is a significant factor in antibacterial activity of the composite CA-MH nanofibrous mats with a p-value of < 0.05.

MH bioactivity is largely associated with MGO content. MH has been reported to reduce the swarming and swimming motility of bacterial strains. It has been reported that MGO in honey cause the loss of fimbriae and flagella in bacteria cell, these protruding structure from the bacterial cells are responsible for bacterial adherence to their host and their propulsion in any medium. MGO bind directly to fimbriae and flagella causing loss of structural integrity and subsequently function [54].

The antibacterial efficacy of CA-MH nanofibrous mats against *Escherichia Coli* was better than against *Staphylococcus Aureus.* This may be due to the difference in the cell wall structure of the gram-negative and gram-positive bacterium [11,31].

Table 2-2 Inhibition zone of CA-MH nanofibrous mats against *S. aureus* and *E.coli*.

Sample labels	Zone of Inhibition (mm)	
	Staphylococcus Aureus	*Escherichia Coli*
CA	0	0
CAH1	1.37±0.37	2.1±0.31
CAH2	2.24±0.23	2.9±0.39
CAH3	2.74±0.24	3.6±0.35

Table 2-3 Bacteria reduction percentage of CA-MH nanofiber mats using OD method.

Sample labels	Reduction (%)	
	Staphylococcus Aureus	*Escherichia Coli*
CA	0	0
CAH1	56.87±2.57	64.55±1.73
CAH2	63.07±0.28	70.19±1.42
CAH3	73.25±0.91	79.98±0.66

Figure 2-10 Antibacterial efficacy of CA and CA-MH composite nanofibrous mats by agar disc diffusion test (AATCC-147 1998).

Figure 2-11 OD measurements for antibacterial efficacy of CA-MH nanofibrous mats against E. coli and S. aureus.

Table 2-4 Minitab analysis of variance (ANOVA) for antibacterial activity of composite CA-MH nanofibrous mats against E.coli and S. aureus.

Parameter	Source	DF	SS	MS	F	p-value
Antibacterial activity						
E. coli	MH content	3	11883.3	3961.09	2882.84	0.000
	Error	8	11	1.37		
	Total	11	11894.3			
S. aureus	MH content	3	9746.27	3248.76	1720.96	0.000
	Error	8	15.10	1.89		
	Total	11	9761.37			

*p-value < 0.05 at α = 0.05; DF – degree of freedom; SS – sum of squares; MS – mean squares

2.3.9 Cell viability

High biocompatibility is expected of an ideal wound dressing. WST-1 chromatic assay was employed to analyze the cytocompatibility of the electrospun composite CA/MH nanofiber mats in contact with NIH 3T3 fibroblast cells for 1, 3, 5, and 7 days. Fig. 2.12 illustrate the cell viability of the neat CA and composite CAH1, CAH2, and CAH3 nanofiber mats. The results showed moderate viability in the range of 51% - 80% for all the specimens in a short period of time 1 to 3 days (control sample showed 100%). For a longer period, the composite nanofiber mats showed higher cell viability reaching to the range of 77% to 134% (control sample showed 100%), indicating good biocompatibility of the produced nanofiber mats. Furthermore, it can be concluded that the addition of MH in the composite nanofiber mats improves the biocompatibility of the CA nanofiber mats.

Figure 2-12 Cell viability of NIH3T3 fibroblast cells cultured for 1, 3, 5 and 7 days in presence of nanofibrous mats.

Figure 2-13 SEM micrographs of NIH 3T3 cells grown on different nanofibrous mats (a) CA, (b) CAH1, (c) CAH2, and (d) CAH3.

To further establish the biocompatibility of CA-MH nanofiber mats, the NIH 3T3 cells morphology on the nanofibrous mats after 3 days of culture was studied (Fig 2.13). Fusiform profile of cell with strong interaction with the nanofiber mats can be seen in Fig. 2.13 (a) neat CA and (b) MH 10 % (CAH1). Increasing the MH content to 15 % and 20 % results in increased spreading of the cell on the mats. Particularly, at 20% MH (CAH3), cells can be seen to spread into the mat structure. From this observation it can be suggested that inclusion of MH in the CA nanofiber improve its affinity for NIH 3T3 cells.

2.3.10 Loading efficiency and release behavior

CA-MH nanofiber mats were evaluated for drug entrapment efficiency using UV-Vis spectrophotometer at maximal spectral absorbance wavelength of MH (275 nm). The nanofiber mats showed an entrapment efficiency of 94.14% for CAH1, 93.38% for CAH2, and 90.47% for CAH3. Higher the entrapment efficiency the better is the system suitable for drug delivery applications. However, 100% entrapment can only be achieved by a core-sheath nanofiber structure [55]. The release behavior of the composite CA-MH nanofiber mats loaded with different quantity of MH are shown in Figure 14 (a).

Figure 2-14 (a) In vitro release behavior of CA-MH nanofibrous mats in PBS solution at pH 7.4 and 37°C (b) Fitted line plot for MH in PBS showing the regression model and coefficient (R^2).

The nanofiber mats with varying quantity of MH ranging from (10 – 20 wt %) showed a similar release behavior. The release profile shows that the MH cumulative release was 17.4, 18.7, and 20.3 % for CAH1, CAH2, and CAH3 at time 5 h. This indicates that there was no burst release during this initial stage. The release slowed down at 30 h time interval and become steady at 35 h interval and remained steady until 45h. At 45 h the cumulative release was 76.78, 80.57 and

81.09 % for CAH1, CAH2, and CAH3 respectively. The porosity and the high surface area of the composite CA-MH nanofiber mats allows the Manuka honey to diffuse into the PBS media. Moreover, the high cumulative release at 45 h may be due to the degradation of the CA at the pH of 7.4 for the PBS medium of release as reported by Milovanovic et al [20].

2.3.11 Antioxidant analysis

An *in vitro* evaluation of the antioxidant potential of the CA-MH nanofibrous mats was done by DPPH free radical scavenging. DPPH assay is based on 2, 2-diphenyl-1-picryl hydrazyl radical for determining radical scavenging activity of the phenolic content of the manuka honey. The DPPH in it radical form has a purple color. Upon accepting an electron from the antioxidant compound, the purple color of the radical turns to yellow. The difference in color of the diphenyl-picrylhydrazine radical was estimated colorimetrically using UV-Vis spectrophotometer [56].

Antioxidant activity benefits to control the overproduction of the reactive oxygen species, which are strongly associated with wound excessive inflammation and leads to chronic conditions [57]. Figure 15 shows the antioxidant activity percentage of DPPH with neat CA and CA-MH nanofiber mats. The neat CA mat has slight radical scavenging activity due to presence of the hydroxyl groups. In composite CA-MH nanofiber mats a regular increase in the radical scavenging can be seen with time and amount of the MH content in the fibers. DPPH was quenched by MH loaded nanofibers in a time dependent manner. For a particular fiber mat the DPPH scavenging rate increased over time. At a particular time, it is evident from the results that as the MH content increases the antioxidant activity of the nanofiber also increases. The nanofiber mat with 20% MH content (CAH3) shows the highest antioxidant activity, 47.25 ± 1.15 at 12 h time interval. As the contact time of the nanofiber mats with DPPH solution increases, more MH was released from the nanofiber mats and dissolved in the solution, thus increasing the DPPH scavenging. Fig. 2.16 portrays the DPPH solutions after 12 h of immersion time with the nanofiber mats with different MH content. The color of the solution without nanofiber mats remained unchanged i.e., purple, while with the increase of the MH content in the CA-MH nanofiber mats, the DPPH solution gradually faded to light pink and yellow, indicating the free radical scavenging activity of the CA-MH nanofiber mats.

Figure 2-15 DPPH scavenging activity of the composite CA-MH nanofiber mats.

Figure 2-16 DPPH solution after 12 h of reaction time with CA-MH nanofibers.

2.4 Conclusion

In current research, nanofibrous mats with different quantity of manuka honey in cellulose acetate were successfully fabricated by employing electrospinning for potential wound care application. The continuous and bead-free nanofibrous mats with well-defined morphology were proven by SEM micrographs. ATR-FTIR fingerprints justified the inclusion of the MH in the composite CA-MH nanofibrous mats. XRD diffractograms discovered that the crystal structure of the CA deteriorates upon the addition of the MH. The produce MH loaded CA nanofiber mats have acceptable mechanical properties. BET analysis showed that the surface area of the fabricated mats decreased with the increase in the nanofiber diameter due to the inclusion of MH. Moreover, the porosity of the composite mats are on the high side which is considered good for gaseous and vapor transport between the wound surface and the ambient environment. WVTR of the produced CA-MH nanofiber mats also fall in acceptable range for wound care. While addition of MH in CA resulted in a decrease in the water contact angle of the resultant CA-MH composite nanofibrous mats, thus showing an increase in the hydrophilicity of the fabricated nanofibrous mats. Cell culture analysis by WST-1 assay revealed that the developed nanofibrous mats showed excellent cell proliferation and growth against the tested NIH 3T3 fibroblast cell line, endorsing good biocompatibility of the electrospun composite CA-MH nanofibrous mats for wound care applications. Furthermore, the composite nanofibrous mats showed acceptable antimicrobial activity against the gram-positive S. aureus and gram-negative E. coli bacterial strains. Antioxidant activity of the CA-MH nanofiber showed that the nanofiber ability to scavenge DPPH radical is dependent on time of contact and the amount of the MH content in the nanofiber mats.

Based on the above in vitro results, the electrospun composite CA-MH nanofibrous mats show high efficacy to prevent bacterial growth on the wound surface. Besides, the nanofibrous mats have high porosity suitable for wound breathability and shows high cytocompatibility to effectively promote wound healing. The future prospect of the undergoing research would be to perform detailed in vivo study on animal model to further promulgate the efficacy of the developed nanofibrous mats as a potential wound dressing.

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Chapter 3

Bioactive Sambong Oil-Loaded Electrospun Cellulose Acetate Nanofibers: Preparation, Characterization, and in-vitro Biocompatibility.

3.1 Introduction

The human history of wound care over the millennia is a combination of trials and errors. Over the past few decades, antibiotics have been the primary source for the treatment of infected wounds. The problem associated with the use of antibiotics is that they only prevent the wound from getting infected instead of speeding tissue regeneration. Furthermore, microbes are constantly evolving and generating immunity against our weapon of antibiotics. Pathogen's mutation ability has outrun the scientific inventiveness in creating new antibiotics and reverted the medical practitioners and pharmaceuticals to investigate the once-forgotten natural approaches for wound care [1].

The wound healing process, triggered by the skin's damage, consists of multiple phases: hemostasis, inflammation, proliferation, and maturation/remodeling [2]. All these four overlapping phases for the reconstruction of healthy skin are needed to complete. Bacteria present on the human skin flora can cause infection and impede the wound healing process [3]. For this purpose, a wound dressing with antibacterial properties can provide a suitable barrier for the pathogens. Different wound dressing materials are used depending on the type of wound and targeting different wound healing phases. An ideal wound dressing is characterized as capable of maintaining a moist micro-environment on the wound/dressing interface, have adequate gaseous and vapor transport, having mechanical strength for clinical handling, provision of barrier properties, biocompatible, non-allergic, and easy to change without trauma [4].

Nanotechnology can be unpretentious sustenance in avoiding the problems associated with high systemic and topical drug doses to prevent infections and allows for manipulating the drug carrier to molecular scale for better interactions with the wound microenvironment [5]. Ultrafine nanofibers produced by electrospinning have gained much interest due to their high surface to volume ratio, porosity, flexibility, and ease of functionalization. They provide a unique avenue for the delivery of therapeutic agents to the wound site. The high aspect ratio leads toward high mass transportation, which is suitable for drug delivery applications. Fibers produced by electrospinning have found their applications in numerous fields such as wound healing [3,6], drug delivery [7], tissue engineering [8], antibacterial [9,10], sensors [11], filtration [12], and other environmental applications [13]. Electrospinning technique is evolving fast from monoaxial [14] to coaxial [15], side-by-side [16], tri-axial [17] and complicated processes [18], and the created nanostructures include core-sheath [19], janus [20], tri-layer core-shell [21] and other complex one [22]. However, the functionalization of nanofibers by adding active ingredients in them in the form of homogenous

distribution all over the polymer matrix [23], or in a separate [24], gradient [25], discrete manner [26]. Among those loading manners, uniform distribution through a single-fluid blended process is still the mainstream [27]. High versatility and flexibility of electrospinning process offer unique advantage of producing continuous and uniform nanofibers and fabrics with controlled morphology and multidimensional structures ranging from one dimension to three dimensions of almost any synthetic and many natural polymers [28]. Electrospun nanofibers are ideally suited as scaffolds for wound healing and serve as a brilliant carrier system for bioactive ingredients, providing spatial and temporal control over biomolecules and their release, thus avoiding systemic exposure and boosting the efficiency of the bioactive ingredient [29].

Chronic wounds like diabetic ulcers and burn are complex wounds and mainly involve slow wound healing rates and require high hospitalization costs. They are thus affecting both the patients and the health care system adversely. The antibiotic resistance has also imposed a severe threat over the use of synthetic medicines. Natural products such as aloe vera, honey, and especially essential oils have mainly gained importance as bioactive agents. All essential oils are particularly volatile and are easily degradable: incorporating them in the nanocarriers should enhance their stability and improve their antibacterial activity [30]. Essential oils obtained from the aromatic plants are composed of a variety of components such as phenols, terpenoids, fragrant, and aliphatic components [31]. From prehistoric times essential oils are vastly used as antibacterial, antiviral, insecticidal, and many other medical applications such as analgesic and anti-inflammatory.

Blumea Balsamifera (BB) is an important medicinal plant from the genus Blumea. It is also known as “Sambong” and is being used as an herbal medicine for thousands of years in Southeast Asian countries like Malaysia, Vietnam, China, and Thailand [32,33]. The essential oil extract from the leaves and stems of the plant is used to treat dermatitis, lumbago, beriberi, eczema, wounds, and also as an insecticide [34]. The extract of the plant has reported possessing antioxidant, antifungal, antibacterial, and antitumor properties [35]. The leave extracts of BB contain considerable amounts of flavonoids and volatile oils [36]. Volatile oil analysis by GC-MS revealed that the L-borneol (33.2%), caryophyllene (8.24%), Iedol (7.12%), and D-camphor (17.76 %) are the most abundant components [37]. L-borneol is an essential ingredient used in healing burn wounds by Chinese herbal medicine practitioners. It has reported fastening wound healing by stimulating the expression of cytokines and growth factors and influencing the anti-inflammatory responses [38]. Pang *et al.* reported that using BB oil externally on the wound model in mice promoted capillary

maturation, collagen deposition, granular tissue formation, and wound contraction [39]. Wang *et al.* reported BB oil was not allergic to the intact skin of the guinea pigs and had no acute toxicity to the rats in both intact and damaged skin models [40].

Cellulose Acetate (CA) is an acetate ester derivative of the natural Cellulose. Cellulose serves as the primary component of the cell walls of the plants and is the most abundant biopolymer on earth [41]. The applicability of Cellulose in the biomedical field is hindered by its limited solubility in organic solvents and its disability to melt due to excessive inter and intramolecular hydrogen bondings. CA is generally produced by acetylation of hydroxyl groups in the cellulose ring. The solubility of the CA is dependent upon the degree of acetylation. CA offers essential advantages such as renewability, affordability, and abundant availability. Its solubility in organic solvents make it an ideal material for electrospinning. CA has found its potential applications in the biomedical field due to its biocompatibility, stability in an aqueous medium, biodegradability, mechanical performance, low cost, and readily availability [42]. Its high affinity for other substances and regenerative properties have made this polymer highly suitable for applications like wound dressings and dermal substitutes. Among various applications of CA, its utilization as a carrier for the transdermal drug delivery is of significant importance due to its ability to remain stable in aqueous mediums [43]. Bioactive agents can be mixed with the polymer solution and produced into a core-shell structure or maybe surface-immobilized by chemical or physical processes. Igor *et al.* reported that CA can effectively prolong the retention of essential oils and can serve as a suitable carrier of these volatile active compounds [44]. Chantarodsakun *et al.* report the production of gingerol loaded CA nanofibers for the controlled release. They stated that ~97% of gingerol released from the CA fiber in acetate buffer solution. The diffusion mechanism mostly governs the release. The released gingerol showed antioxidant activity, and the gingerol was weakly cytotoxic [45].

Patrojanasophon *et al.* [46] reported the release of water-soluble drug from CA nanofibers depending upon the degree of alignment of the nanofibers on the collecting mandrel. They found out that randomly oriented nanofibers showed controlled release due to formation of an entangled mesh network affecting the diffusion of drug to the external medium. While highly oriented nanofiber showed an abrupt release. So, by manipulating the orientation of nanofibers one can design and abrupt or controlled release CA nanofiber-based drug delivery system. Wutticharoenmongkol *et al.* [47] fabricated Gallic acid loaded CA nanofibers with 10 – 40 % by

weight of Gallic acid. The nanofibers showed antibacterial efficacy against *S. Aureus* and retained it antioxidant activity in both as spun and as released condition, implying that the gallic acid loaded in the CA did not lost it bioactivity. Liu *et al.* [48] prepared rectorite and paclitaxel immobilized cellulose acetate nanofibers. They reported that the CA served as an effective polymer for loading of rectorite and paclitaxel. Furthermore, the addition of rectorite improved the immobilization of paclitaxel in the CA nanofibers and have synergistic cytotoxicity effect on the gastric cancer cells (SGC7901). Hua *et al.* [49] fabricated the pH responsive polyurethane/core and cellulose acetate phthalate/shell nanofiber for intervaginal drug delivery. These core shell fibers showed improved mechanical strength as compared to the neat cellulose acetate phthalate. The cellulose acetate phthalate imparts pH responsiveness to the core-shell structure giving the fibers potential for "semen sensitive" (intravaginal) drug delivery. Pereira *et al.* developed chitosan nanowhiskers coated CA electrospun nanofibers. The coated nanofibers were effective against gram negative bacteria and were atoxic to healthy Vero cells [50].

Liakos *et al.* reported the CA-lemongrass essential oil nanocapsule. The nanocapsules adhered to the mucous membrane well and were effective against *Escherichia coli* (E.Coli) and *Staphylococcus aureus* (S. Aureus) [51]. Vongsetskul *et al.* report CA nanofibers loaded with A. ebracteatus Vahl. ~99% of the loaded extract was released by diffusion in acetate buffer solution and was active with antioxidant activity along with moderate toxicity [52]. Liakos *et al.* reported the rosemary and oregano oil loaded CA nanofibers. They stated that the CA electrospun pads with 5% essential oil were effective against bacterial and fungal strains [53]. In another work, Liakos *et al.* reported grafting of peppermint, cinnamon, and lemongrass on the CA chain and produced antibacterial and antifungal nanocapsules [54]. Fawal *et al.* reported rosemary and aloe vera essential oils loaded CA films. They reported the effectiveness of the films against E.Coli and *Bacillus subtilis* (B. Subtilis). The inclusion of essential oils also improved the antioxidant activity of the CA films [55].

The aim of this study was to construct various amounts of BB oil loaded CA nanofibers intended to have high potential as bioactive wound dressing material. In this way, we studied the effect of various concentrations of BB oil (10%, 15%, and 20%) on the surface morphology, fiber diameter, porosity, antioxidant, antibacterial, cell viability, release behavior, mechanical, and chemo-physical, along with surface wettability, vapor transport rate, water absorption and weight loss properties of CA nanofiber mats. To the best of our knowledge to date, there is no work reported

relating to the incorporation of BB oil in any polymeric system, whether nanocapsules, films, sheets, or nanofibers. This work would be the first of its kind about the inclusion of BB oil in CA nanofibers by electrospinning technique.

3.2 Experimental

3.2.1 Materials

Cellulose acetate (~40% acetyl substitutions by weight content), MW 30000 g/mol purchased from Sigma Aldrich (USA). Acetone (99.5 %) an N-N-Dimethylformamide (DMF, 99.8%) were procured from Fujifilm Wako Chemical Ltd. (Japan). Blumea Balsamifera (BB) oil obtained from Shaanxi Bolin Biotechnology Co. Ltd, China. All chemicals are used as received without any further purification.

3.2.2 Electrospinning solution preparation

All solutions for electrospinning were made in a DMF/Acetone (55:45) solvent. The pristine CA solution was made up to 20% by weight. For BB oil loaded solutions, BB oil was added in 10, 15, and 20% by weight concerning CA content. The spinning solutions were agitated 1200 rpm for 24 hours to ensure complete dissolution and homogeneity. The solutions/samples produced were labeled as CA, CA/BO10, CA/BO15, and CA/BO20.

3.2.3 Electrospinning process

Electrospinning involves the usage of a strong electric field applied across a capillary carrying the spinning solution and a collector. When the applied electric field overcomes the surface tension of the polymer solution, a jet is formed. The jet draws during it flow towards the collector and thins down to the nanoscale, and the solvent gets evaporated during the flight period. In this research, a horizontal electrospinning module was set up by using a high voltage power supply (Har-100*12, Matsusada Co., Tokyo, Japan) and a syringe pump (KDS-100, KD Scientific, USA). The solution was filled in a 25 ml syringe equipped with a 20 gauge needle. A high voltage of 14 kV was applied across the needle and the collector covered with butter paper. The flow rate was kept at 0.5 ml/h, and a collector to needle distance was 180 mm. Electrospinning was carried out at 25°C and relative humidity of ~45%. All samples were spun for 36 h to ensure a uniform thickness of the fibrous mats.

3.2.4 Characterization

The morphological study of the produced fibers was studied with scanning electron micrographs using a scanning electron microscope (SEM, JSM-6010LA, JEOL, Japan). Fibrous mats sputtered coated with platinum before subjecting to SEM imagery. The fiber diameter was analyzed using ImageJ software (version 1.4.3).

A Fourier Transform Infra-red (FTIR) spectroscopy (Prestige-21, Shimadzu, Japan) was used to determine the physicochemical analysis of the fibrous mats. FTIR spectra were scanned over the wavelength range 4000-400 cm^{-1} at room temperature, and a spectrum was accumulated over 20 scans.

The X-ray diffractograms (XRD) of the neat and BB oil loaded fibrous mats were determined by an X-ray diffractometer (Miniflex 300, Rigaku Co., Ltd., Japan) operating at 30 kV and 500 mA. Diffractions were detected in the $2\theta = 5°- 80°$ range with a step of 0.05°.

The mechanical performance of the produced fibrous mats was analyzed by subjecting them to tensile tests using the universal tensile testing machine (UTM), Tensilon RTC 250A, A&D Company Ltd. Japan. Stress, strain, and elastic modulus was determined from the obtained UTM data.

3.2.5 Porosity

For porosity, the fibrous mats were cut into 4 cm × 4 cm and weighed. The thickness of the mats at five different places was measured using a digital multi-meter. The apparent volume (V_a) was measured from the previously mentioned dimensions. The theoretical volume (V_g) was measured by the densities of the CA (1.3 g cm^{-3}) and BB oil (0.989 g cm^{-3}) [56] and their respective weight ratios in the samples. Porosity was measured by the following Eq. 1 [3].

$$Porosity\ \% = (1- V_g/V_a) \times 100 \quad (1)$$

3.2.6 Antibacterial assessment

The AATCC 147-1998 standard test method was employed to qualitatively assess the antibacterial activity of the BB oil loaded fibrous mats [57]. A 100µl of 10^{-5} dilution of overnight cultured E.coli and S.aureus were spread over the agar plates. The fiber mats were UV sterilized for 6 hours before subjecting to antibacterial test. The fiber mat samples (10 mm, diameter) were gently placed over

the agar plates and incubated overnight. The agar plates were examined for bacterial growth interruption underneath and along the border for the zone of inhibition.

Quantitative assessment for antibacterial efficacy was made by optical density (OD) technique, as described elsewhere [58]. Both E.coli and S. aureus were cultured in 100 ml nutrient broth. A 10^{-4} dilution was prepared, and 10µl of it was dispensed in 10ml of fresh nutrient broth solution. 50 mg of each fibrous mat was added to their respective bottles and incubated for 24 h. The bottles without fibrous mats were considered to be controls. After 24 h, the absorbance of the nutrient broth solutions was measured at 600 nm using a UV-Vis spectrophotometer (Lambda 900, Perkin-Elmer, USA). The bacterial reduction was measured in percentage using Eq. 2.

$$Bacterial\ reduction\ (\%) = \frac{Absblank - Abssample}{Absblank} \times 100 \quad (2)$$

Where "Abs_{blank}" is the absorbance of the blank bottle and "Abs_{sample}" is the absorbance of bottles containing fiber samples.

For bacterial reduction with the time of contact, the absorbance of the sample bottles at time intervals, 2, 4, 6, 8, 10, and 12 h were taken compared with the blank bottle absorbance, and percentage reduction was calculated according to Eq. 2.

3.2.7 Cell proliferation (WST-1 colorimetric assay)

The WST-1 colorimetric assay was employed to determine the cell proliferation of the NIH 3T3 mouse fibroblast cell in contact with the BB oil loaded CA fibrous mats. Briefly, NIH 3T3 cells were cultured in Dulbecco modified eagle medium (DMEM) accompanying 10% fetal bovine serum (FBS) in an incubator with 5% CO_2 environment. The fibrous mats were sterilized in ethanol and washed with phosphate buffer saline (PBS) solution. The mats were placed in 96 well culture plates. The wells without mats were considered as control. The wells were seeded with 1×10^3 cells per well. The proliferation was assessed at 1, 3, 5, and 7 days interval. At each corresponding day, 10µl WST-1 was added to each well and incubated for two hours. Absorbance of control and sample was measured at 440 nm using a microplate reader (Thermos scientific, Multiskan FC instrument). The cell viability was measured according to Eq. 3. Readings were taken in triplicate.

$$\text{Cell viability}\ (\%) = \frac{Abssample}{Abscontrol} \times 100 \quad (3)$$

3.2.8 Antioxidant assay

2, 2-diphenyl-1-picryl hydrazyl radical (DPPH) free radical scavenging assay was performed to determine the antioxidant activity of the fibrous mats. A 300 mg of each fibrous mats were immersed in 3 ml of 0.1 mM DPPH methanol solution and incubated for different periods of time (2, 6, and 12 h) at 37ºC under dark conditions. DPPH solution without sample was considered as control. At the specific time interval, the absorbance at 517 nm was measured using a UV-Vis spectrophotometer (Lambda 900, Perkin –Elmer, USA). The antioxidant activity was measured in percentage using Eq. 4.

$$Antioxidant\ activity\ (\%) = \frac{Acontrol - Asample}{Acontrol} \times 100 \quad (4)$$

Where A_{sample} and $A_{control}$ is the absorption of DPPH with and without fibrous mats.

For measuring half inhibition concentration (IC_{50}) of the BB oil for DPPH assay. Different concentrations of BB oil in methanol were prepared as: 1, 3, 5, 10, 20, 30, 40, 50, and 100 mg/ml. 1 ml of BB oil respective stock solution was added to 3 ml of 0.1 mM DPPH in methanol and incubated at 37ºC in the dark for 30 minutes. After 30 minutes, the absorbance at 517 nm was measured using a UV-Vis spectrophotometer (Lambda 900, Perkin –Elmer, USA), and the percentage of free radical inhibition was calculated as per Eq. 4.

3.2.9 Moisture vapor transport rate (MVTR)

To evaluate the MVTR of the electrospun fibrous mats, ASTM E96-95 was employed. The fibrous mats were cut into a circle of 90 mm and placed over a cup of 70 mm diameter. The containers were prefilled up to three quarters with distilled water. The cups were placed in an incubator at 37ºC and 40% humidity. The cups were weight both before and after putting in an incubator, and weights were recorded as W1 and W2. MVTR was measure by Eq. 5.

$$MVTR = W1 - W2/At \quad (5)$$

Where “A” is the area of the cup mouth, and “t” is the time in which “W1-W2” happened.

3.2.10 Release Profile

The release of the BB oil from the fibrous mats was measured by the total immersion method into PBS (pH ~7.4). The maximum absorption wavelength (272 nm) of BB oil in PBS was determined from spectra over wavelengths ranging from 200 to 700 nm, which is close to the previously reported literature [59]. The calibration curve of the BB oil was prepared in PBS using a UV-Vis

spectrophotometer (Lambda 900, Perkin-Elmer) at 272 nm (λ_{max}). Fibrous mats (50 mg) were dipped in PBS solution at 37°C for time intervals at 0 to 72 h. At the respective time intervals, 1 ml of the solution was extracted, and the stock was replenished with the same amount to keep the settings constant. Fresh PBS with suitable pH was used to replace the withdrawn sample volumes. Sink conditions were assured following sample withdrawal and dilution due to replacement media. The absorbance spectra of the BB oil at certain time intervals were converted into concentration using the calibration curve. The experiment was conducted in triplicate, and the results were represented as averages with standard deviation.

3.2.11 Water contact angle, absorption, and weight loss analysis

The surface wettability of the composite nanofibers was analyzed by water contact angle using Digidrop, GBX, France, contact angle analyzer. A static water contact angle was calculated using 28-gauge needle. Five readings for each type of nanofibers were calculated and average was recorded.

Water absorption was measured as stated elsewhere [60]. A pre weighed (W_d) sample was immersed in distilled water at 37°C for 24 h. After the required time the sample was taken out, left to drain hung in air and then wiped with filter paper to remove the surface water, and weighed (W_w) again. The water absorption of the nanofibers was calculated as per Eq. 6.

$$Absorption = \frac{Ww - Wd}{Wd} \times 100 \quad (6)$$

For weight loss measurement pre weighed (W_i) sample were immersed in distill water for 24 h. Afterwards, the samples were dried in an oven at 50°C for 24 h and weighed (W_f) again. The percentage weight loss was determined from the Eq. 7.

$$Weight\ loss\ \% = \frac{Wi - Wf}{Wi} \times 100 \quad (7)$$

3.2.12 Statistical analysis

All statistical analysis regression equations and correlation coefficients were carried out using MINITAB 17 ® statistical software [61].

3.3 Results and Discussion

3.3.1 Morphology, diameter analysis, and porosity

SEM images (Fig. 3.1) were analyzed for accounting any difference in morphology and diameter of the BB loaded CA fibers as compared to neat CA fibers. The SEM micrographs show that the bead-free continuous fibers are obtained in all cases. The average diameter for the neat CA fibers was calculated to be ~324±94 nm. The BB oil loaded fibers showed the diameters of around 362±162, 420±178, and 448±180 nm for CA/BO10, CA/BO15, and CA/BO20, respectively. The increase in the fiber diameter can be recognized as the successful encapsulation of BB oil in the ultrafine CA fibrous mats.

A decrease in the porosity of the BB oil loaded CA fibrous mats were reported (Fig. 3.2). This decrease can be attributed to the increase in the fiber diameters due to the inclusion of BB oil in the CA fibers. But the overall porosity of the produced fibrous mats is on a higher side, which facilitates the transport of gasses and vapor between the wound surface and the outer environment [62].

3.3.2 Physicochemical analysis

FTIR spectrums for CA, BB oil, and BB oil loaded fibrous mats are shown in Fig. 3.3 (a and b). A weak broad peak of the – OH group present in CA can be observed at around 3450 cm^{-1}. A twin peak at 2959 and 2917 cm^{-1} are due to the – CH2 stretching. The solitary peak at 1743 cm^{-1} is due to – COO^{-1} group. The peak at 1365, 1236, 1041, and 917 cm^{-1} are because of – CH3, C – O, C – O – C, and C1 – H stretching and twisting. The peaks observed are in accordance with the reported literature [3,63–65].

In the FTIR spectra of BB oil, an – OH peak at around 3500 cm^{-1} was observed, which is due to alcohol present in the borneol [66]. The sharp peaks between 3000 and 2800 cm-1 are due to the – C – H groups of the hydrocarbon alkanes of monoterpenes in the BB oil. (– C = O) aliphatic ketone group in camphor was observed at around 1750 cm^{-1}. – C – O – C – strained ether grouping from the 1,8 – cineole can be observed at 1035 cm^{-1}. The strong peak ~980 cm^{-1} is due to the out of phase twisting of the – C – H from isoprenoids [67].

Figure 3-1 SEM micrographs and diameter histograms of CA (a), CA/BO10 (b), CA/BO15 (c), and CA/BO20 (d).

The sharp peaks between 3000 and 2800 cm^{-1} of the hydrocarbon alkanes and a little shift in the peak – OH from 3450 to 3500 cm^{-1} in the BB oil loaded CA fibrous mats are the indication that the oil is successfully entrapped in the CA nanofibers after electrospinning. Besides that, there is no significant difference observed in the FTIR spectra of the BB oil loaded CA fibrous mats.

Figure 3-2 Porosity analysis of the neat and BB oil loaded ultrafine CA fibrous mats.

3.3.3 XRD

Fig. 3.4 represents the XRD diffractograms of the neat and BB oil loaded ultrafine CA fibrous mats. It is an established fact that additive mixing in pure material results in a change in its molecular packing [68]. CA shows two broad peaks at $2\theta = 10^{o}$ and 19° because of the regular aligned packing of its amorphous microstructure and coincides with the previously reported literature [3,65]. The addition of BB oil in the fibrous mats could have causes a change in the polymer chain packing in the pure CA nanofibers and thus results in a decrease in the intensity of the peaks. This change can

Figure 3-3 FTIR spectrum of (a) CA, and BB oil and (b) BB oil loaded ultrafine fibrous mats.

be attributed to the shift in inter and intra-polymer chain hydrogen bonding of the hydroxyl groups present in the CA polymer. The decrease in the peak intensity can be a result of the misalignment of the polymer microstructure [3,69,70].

Figure 3-4 XRD diffractograms of neat and BB oil loaded ultrafine CA fibrous mats.

3.3.4 Mechanical performance

Adequate mechanical strength and flexibility is an important feature for wound dressing in clinical handling and wound healing. The skin mechanical properties varies from 0.1—32 MPa for strength and 0.42 – 2.26 for strain ration at break [71]. So material intended for the skin regeneration purposes should have properties that coincide with skin properties. But the skin structure possess heterogeneity and the above value are not absolute and are dependent on several factors like genetics, age, and color of the skin [72]. The Fig.5 gives the stress-strain diagram of the neat and BB oil loaded ultrafine Ca fibrous mats. The maximum stress of the neat CA mats was ~ 2.6 MPa, and that of BB oil loaded mats was 1.2, 1.09, and 0.87 MPa for CA/BO10, CA/BO15, and

CA/BO20 respectively. The decrease in the strength of the fibers can be attributed to the misalignment of the polymeric chains. This misalignment could lead to the change in the hydrogen bonding of the hydroxyl groups present in the CA. Interruption in the hydrogen bond formation can be a reason for the decrease in the strength of the CA fiber and had been stated while encapsulating other essential oils in the CA nanofibers [45].

Figure 3-5 Stress-strain diagram of the neat and BB oil loaded ultrafine CA fibrous mats.

3.3.5 Antibacterial efficacy

The antibacterial efficacy of the neat and BB oil loaded ultrafine CA fibrous mats were judged against the *S.aureus* and *E.coli*. The chosen strains are the most common infection-causing bacteria to the soft tissues and responsible for delayed wound healing [3]. Fig. 3.6a shows the halos generated by interruption of bacterial growth around the fibrous mats. The halos (zone of inhibition) were measured using ImageJ software and are recorded in millimeters and are given in Table 3.1. It is evident from Fig. 3.6a and Table 3.1 data that as the amount of BB oil in the CA

fibers increases, the halo diameter increases. The largest zones of inhibition were measured for CA/BO20 with 3.4 and 3.8 mm for E.coli and S.aureus, respectively.

Table 3-1 Antibacterial efficacy (Halo zones) of BB oil loaded ultrafine CA fibrous mats.

Sample labels	Zone of Inhibition (mm)	
	E.coli	***S.aureus***
CA	0	0
CA/BO10	1.8±0.15	1.16±0.17
CA/BO15	2.1±0.38	2.47±0.59
CA/BO20	3.4±0.28	3.8±0.35

For the quantitative assessment of the antibacterial efficacy of the produced nanofiber mats, the OD technique was employed. Fig. 3.6b shows the percentage reduction in the bacterial growth in contact with BB oil loaded samples at 24 h time interval. The same trend of increasing the BB oil concentration in the fibrous mats resulting in a decrease of bacterial growth was observed. Fig. 3.6c shows the reduction in the bacteria in the broth medium as a function of time of contact. The two trends are evident from Fig. 3.6c one as the amount of BB oil in CA fibers increases the bacterial growth decreases, and second, as the time of contact of the fibrous mats in the bacteria medium increases the bacterial inhibition increases. This second trend of the time of contact can be attributed to the fact that the release of BB oil from the fibrous mats is time-dependent and not abrupt. The mechanism of action of the BB oil is primarily associated with its ability to disrupt the bacterial cell wall and changes its permeability [73]. Most of the essential oil are more effective against the gram-positive bacteria than the gram-negative ones. This difference may be due the difference of the cell wall composition. On average 90 – 95% of the gram positive cell wall consist of peptidoglycans, which allows hydrophobic molecules to penetrate the cells and act on cell wall and within the cytoplasm. Phenolic compounds, constituents of the essential oil penetrates the gram positive bacterium cell walls and perform their antibacterial activity either by inhibiting enzymes of energy production or denature the proteins in the cell wall thus damaging the cell wall barrier [74,75].

Figure 3-6 Antibacterial efficacy of BB oil loaded ultrafine CA fibrous mats: (a) zone of inhibition, (b) OD after 24 hour of incubation in bacterial medium, and (c) bacterial reduction as a function of time of contact.

3.3.6 Cell viability

Cell viability of the NIH 3T3 mouse fibroblast cell line in the presence of BB oil loaded CA fibrous mats were studied by employing ISO 10993-5 [76]. According to ISO-10993-5, cell viability above 80% is considered to be non-toxic, between 80-60% weakly toxic, 60-40% moderately toxic, and below 40% highly toxic. Fig. 3.7 shows the cell viability of the NIH 3T3 cells with BB oil loaded CA fibrous mats at 1, 3, 5, and 7 day time intervals. For 1 to 3 days, the cell viability of the neat and bb oil loaded samples remained moderate, i.e., 50 – 75 %. For the more extended periods of incubation 5 to 7 days, the cell viability of all samples was in the range of 75 – 95 %, with CA/BO20 showing the best cell viability of ~92% and thus rendering the produced BB oil loaded CA fibrous mats non-toxic. From the results in Fig. 3.7 it can be deduced that the addition of BB oil has not hampered the biocompatibility of the CA fibrous mats. Table 3.2. provides a comparison of bioactivity and biocompatibility of the previously reported works.

Figure 3-7 Cell viability % of NIH 3T3 cell line with neat CA and BB oil loaded ultrafine CA nanofibers. Maximum viability at Day 7 by CA 84%, CA/BO10 86%, CA/BO15 87%, and CA/BO20 92% w.r.t control (100%).

Table 3-2 Comparison of bioactivity and biocompatibility of essential oil loaded nanofiber membranes.

Polymer Matrix	Antibacterial Activty		Antioxidant	Cell Viability %	Remarks
	Gram +	Gram -			
Poly vinyl alcohol/Chitosan/Gelatin nanofibers + Zataria multiflora EO [77]	Yes	Yes	--NA--	~90% (Increase in essential oil content decreased cell viability.)	Glutaraldehyde vapor were used to ensure hydro-stability. Difficult to prepare spinning solution in aqueous medium.
Poly lactide-co-caprolactone/ silk fibroin nanofibers + Oregano EO [78]	Yes	Yes	Mild	~65% (weakly toxic at 5% concentration.)	Provides an easy route to encapsulate essential oils. Non adherent to wound surface.
Poly (Ɛ-caprolactone) nanofibers + Peppermint EO [79]	Yes	Yes	--NA--	~98% (EO addition did not affected the cell viability of PCL nanofibers)	Low encapsulation efficiency up to 33 %.
Zein/MMT + H. perforatum oil [80]	Yes	Yes	--NA--	~85% at 7 days incubation	Enhance the fibroblast migration and support wound healing.
Poly (Ɛ-caprolactone)/ gelatin nanofibers + Clove EO [81]	Yes	Yes	--NA--	~98% (EO addition did not affected the cell viability)	Potential candidates for antibiotic-free wound healing applications
Poly vinyl alcohol + palmarosa oil and phytoncide oil [82]	Yes	--NA--	--NA--	--NA--	PVA require additional treatment to be stable in aqueous medium.

CA + Rosemary and Oregan [53]	Yes	Yes	--NA--	--NA--	The Ramman spectrum revealed that R and Or essential oils were efficiently incorporated into electrospun fibers.
CA + Cinnamon, Lemongrass and peppermint [83]	--NA--	Yes	--NA--	Nontoxic to the fibroblast and keratinocytes cell lines.	Essential oils were effectively encapsulated into the CA nanofibers, the nanofibers were effective against *E.Coli*
CA + BB oil (current work)	Yes	Yes	Good	~92% (cell viability increased with BB oil content increase up to 20%)	Easy processing, biphasic release good for acute wound, and stable in aqueous medium, potential

3.3.7 Antioxidant Activity

DPPH free radical scavenging activity was performed to evaluate the antioxidant activity of the neat and BB oil load CA fibrous mats (Fig. 3.8a). The DPPH free radical upon acceptance of an electron from the flavonoids in BB oil changes its color to yellowish, as can be seen in the inset in Fig. 3.8a. This difference was colorimetrically measured using a UV-Vis spectrophotometer, and radical inhibition was measured. CA/BO20 showed the highest antioxidant activity of 68% at 12 h. The neat CA fibrous mat showed a little antioxidant activity due to the presence of – OH group in its polymer chains. In BB oil loaded fibrous mats, there was a sequential increase in the antioxidant activity with the rise in BB oil content in the fibers. Also, an improvement was observed with the increase in the contact time of fibrous mats with DPPH solutions. This rise in antioxidant activity can be justified as the contact time increases more and more BB oil release from the fibrous mats into the DPPH solution; thus a higher free radical scavenging can be observed. The BB oil half inhibition concentration was calculated using the regression equation given in Fig. 3.8b. The IC_{50} value for BB was estimated at 146 mg/ml (146 g/L); this shows that the BB oil used in the current study has a week antioxidant activity, i.e., $IC_{50} > 100$ g/L [37].

3.3.8 MVTR study

Moisture balance on the surface of the wound plays a vital role in wound healing. G.D. Winters in 1963 demonstrated that keeping the surface of the wound moist deliberately can increase the rate of re-epithelialization and thus promotes wound healing [84]. Wound dressing can control this moisture transfer from the surface of the wound and in turn controls the hydration of the wound. The MVTR values of 1800 – 2500 g/m^2/day are considered to be ideal for wound healing [3,85,86]. Over dry wound surfaces due to high MVTR results in scar formation. In contrast, a very low MVTR will result in an over-accumulation of wound exudate and maceration of the healthy skin around the wound and infection. The neat and BB oil loaded CA fibrous mats exhibit MVTR in the range of 2450 – 1750 g/m^2/day, as shown in Fig. 3.9. It is evident that the inclusion of BB oil results in a decrease in MVTR. This decrease could be attributed to the hydrophobic nature of the essential oils and a reduction in the porosity of the fibrous mats due to the addition of the BB oil. The overall MVTR value of the neat and BB oil loaded CA fibrous mats are in the acceptable range (1800-2500 g/m^2/day), as reported in the previous literature [85,86].

3.3.9 Release behaviour

The *in vitro* drug (BB oil) release was carried out in PBS, pH (~7.4), at 37ºC. Fig. 3.10 provides an overview of the release profile from the three formulations used in the study. A biphasic BB oil release profile, shown by orange and green dashed lines in Fig.10, can be observed in all cases over the period of 72 h. The cumulative BB oil percentage of release from the CA fibrous mats (CA/BO10, CA/BO15, and CA/BO20) obtained was 86, 91, and 97%, respectively. The first phase, 0 to 12 h, was a burst release of the BB oil and can be attributed to dissolution and diffusion of poorly encapsulated BB oil in the fibrous mats. The second phase follows the diffusion of BB oil from the polymer matrix. To study the release kinetics of the fibrous mats, the release data was subjected to the zero-order kinetics model (cumulative release % Vs time), first-order kinetics (Log of cumulative drug remaining % Vs time), and Higuchi model (cumulative release % Vs square root of time). The mathematical models and the coefficient of correlations (R^2) are given in Fig. 3.11. According to the highest value of the correlation coefficient (R^2), it is evident that the release of BB oil from the CA fibrous mats follow the first-order kinetics, where the rate of drug release is

Figure 3-8 DPPH free radical scavenging activity of the neat and BB oil loaded CA fibrous mats (inset shows the DPPH solutions at 12 h) (a) and half inhibition concentration (IC50) of the BB oil.

directly proportional to the concentration of the drug undergoing reaction, i.e., higher the amount higher will be the reaction [87].

Figure 3-9 MVTR of the neat CA and BB oil loaded electrospun CA fibrous mats.

The developed system followed the first-order drug release mechanism. That means, initially the drug release was abrupt, and then as the time passed, the release was sustained. This type of release is always required to tackle acute open wounds. As initially, medical practitioners apply a high dose to prevent the infection's possibility. Thus, at start high drug release is required followed by slow release to maintain a contamination-free environment. Thus, the produced systems justify its application in wound dressing. The primary aim of the advanced wound dressing is to prevent infections by bacteria and fungi. The wound dressing should contain bacterial and fungal proliferation and possibly should eliminate all pathogens. The dressings loaded with drug for antibacterial purposes should also do the same by release of the drug to the wound surface and eliminate any bacteria. As to produce an antibacterial wound dressing was one of the primary goals

of this research. The release behavior of the BB oil from the CA nanofiber is quite suitable for the application as an effective antibacterial wound dressing. From the release behavior, it can be seen that the initial release of the BB oil was fast, and with the passage of time, the release become more stable. This release is in accordance with the First-order kinetics, where the drug release is proportional to the amount of drug in the system. So, initially, when the BB oil in the nanofibers was high, the release rate was higher and vice versa. The per unit time release is associated with a constant fraction of drug eliminated from the system. Such an approach is suitable for sustained release of drug fraction per unit time. The burst release of BB oil reduces the pain sensation, while the gradually slow release during the following hours ensures an anti-inflammatory and analgesic effect for a more extended period of time needed for wound healing. Such kinetic profiles with biphasic drug release are targeted for the control of local inflammation and pain associated with a cutaneous wound because the first 12 – 48 h are critical for the wound healing process [88].

Figure 3-10 (a) In vitro release profile of BB oil from CA fibrous mats in PBS, (b) calibration curve of the BB oil in PBS showing regression model and correlation coefficient (R^2).

3.3.10 Water contact angle, absorption, and weight loss analysis

A static water contact angle (WCA) was measured to determine the surface wettability of neat and BB oil loaded nanofibers Fig. 12 a. WCA gives information about the outer layer of the material and is often influenced by the surface structure, roughness, and its composition. The neat CA nanofibers showed a hydrophobic surface (WCA 118°).

Figure 3-11 (a) Zero order kinetic model, (b) First order kinetic model, and (c) Higuchi model of diffusion for the BB oil release in PBS from CA fibrous mats.

This is concurrent with the hydrophobic nature of the cellulose acetate. A high hydrophobicity could be beneficial since it reduces wound dressing to adhere to the wound surface [89]. The addition of BB oil further increases the WCA of the nanofiber mats to 119º for CA/BO10, 126º for CA/BO15, and 130º for CA/BO20. This increase may be attributed to the hydrophobic nature of the essential oil. Not only the hydrophobic nature of the essential oil but also the hierarchical micro/nanostructural morphology of the electrospun mats also adds to the hydrophobicity of the CA nanofibers [90].

Irrespective of the high surface hydrophobicity, the neat CA nanofiber mats showed surprisingly high water absorption (~1250%, Fig. 12 b). This high absorption can be explained by the formation of the hydrophilic area along the fibers as only ~40% of the hydroxyl groups are substituted by the acetate groups (according to the manufacturer's specifications for CA). The remaining 60% serve as channels for water uptake and can be accountable for this high absorption capacity. This micro/nanoporous hydrophilic portion in the nanofiber mats provides for the water retention by capillary action. The incorporation of the BB oil resulted in a decrease in the water absorption capacity of the CA nanofiber mats as the essential oil is hydrophobic and does not allow for water retention by the capillary action. Thus, the water absorption for the BB oil loaded CA nanofibers decreased from 1251% for neat CA to 933% for CA/BO10, 752% for CA/BO15, and 383% for CA/BO20.

All the nanofiber mats showed good stability after water immersion for 24 h, as can be deduced from the very low weight losses for the samples Fig.12 c (~1.5% for CA, ~3.5% CA/BO10, ~5.5% CA/BO15, and ~11.5% for CA/BO20). The weight loss though was small but increased as the amount of BB oil incorporated in the CA nanofiber mats increased. The increase in weight loss can be attributed to the release of BB oil during immersion in water and also evaporation during drying in the oven.

Figure 3-12 The (a) WCA, (b) absorption, and (c) weight loss for neat and BB oil loaded CA nanofibers.

3.4 Conclusion

Ultrafine bead-free continuous CA fibrous mats with three different concentrations of BB oil is prepared for the first time by electrospinning at 14 kV. FTIR spectrum indicated the retention of BB oil in CA after electrospinning. The inclusion of BB oil resulted in a decrease in the tensile strength due to the misalignment of the CA polymeric chains. The increase in fiber diameter due to BB oil inclusion resulted in a reduction of porosity of the mats. The BB oil loaded CA mats showed good antibacterial efficacy against the *E.coli* and *S.aureus* in agar disc diffusion and OD tests. The OD test revealed that the bactericidal effect of the BB oil loaded mats is time-dependent. The BB oil loaded mats showed good cell viability for NIH 3T3 cells with ~92% cell viability at a 7-day time interval. The BB oil showed week antioxidant properties with an IC_{50} value of 146 g/L. The BB oil release from the mats was reported as a biphasic release and followed the first-order kinetics. The MVTR values of the neat and BB oil loaded fibrous mats are in the acceptable range and thus promote adequate gaseous and moisture transport from the wound surface to the outer environment. As the primary focus was on the bioactivity of the BB oil loaded CA nanofibers, it can be inferred from the results that as the BB oil amount increases in the CA nanofibers, the antibacterial activity and cell viability of the nanofibers also increased. Although with the increase of BB oil, the tensile properties and MVTR of the CA nanofiber mats decreased; still, the values are in the acceptable range.

Based on the *in vitro* results, the BB oil loaded CA nanofibers show adequate biocompatibility and high efficacy to contain bacterial growth. Besides, the nanofiber mats have good MVTR and porosity for wound breathability. The future prospect will be to promulgate the efficacy of the BB oil loaded mats by undergoing detailed *in vivo* analysis.

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Chapter 4

"Clay-Corn-Caprolactone" a Novel Bioactive Electrospun Scaffold for Bone Tissue Engineering.

4.1 Introduction

The musculoskeletal system, including bones, muscles, ligaments, tendons, and other connective tissues, support human body weight and related movements. Injuries, diseases, and aging can cause deformation in the musculoskeletal system and lead to trauma and associated pains. Musculoskeletal tissue engineering focuses on mending and regenerating the damaged tissues and bones employing biomimetic scaffolds that closely mimic the extracellular matrix as substitutes. One such approach includes the fabrication of osteoconductive scaffolds that facilitate the development of functional bone tissues [1].

Several methods have been utilized for developing biomaterial scaffolds. Traditional technology includes particulate leaching, solvent casting, phase separation, lyophilization, sol-gel technique, while the more advanced techniques include self-assembly, additive manufacturing, and electrospinning [2]. Electrospinning is of particular interest as it can develop fibrous scaffolds that can mimic the 3D architecture of extracellular matrix, along with specific characteristics of high surface to volume ratio, tunable porosity, suitable mechanical properties, and ability to conform to required shape [3]–[7]. The electrospinning technique provides a very convenient way to incorporate organic/inorganic materials into the polymeric scaffolds. Developing an organic/inorganic electrospun scaffold could be an ideal substrate simulating the natural organic/inorganic bone matrix composed of collagen and apatite [8].

A variety of synthetic and natural fillers are available as reinforcement agents to improve the mechanical properties and bioactivity of the polymeric scaffolds. Clay has emerged as one of the inorganic fillers that can substantially improve the mechanical properties and bioactivity and have been previously studied in processing bone cement [9]–[12]. Illite is a type of non-expanding clay found in temperate and cold regions. It has a dioctahedral 2:1 structure having one octahedral layer sandwiched between two tetrahedral sheets [13]. Illite possesses a large specific surface area and can serve as a promising adsorbent. Most of the previous studies focus on the illite usage as a heavy metal ion adsorbent from the solution [14]. Illite can also adsorb organic moieties such as tetracycline because of its cation exchange capacity and large specific surface area [15]. Based on these properties of Illite, we are hypothesizing that illite addition will improve the hydroxyapatite mineralization over the scaffold. Illite has a long history as a therapeutic agent for various diseases involving cerebrovascular, phlegm dispersing, and activation of blood circulation and Qi-tonifying,

according to Dongui Bogam, the Korean medicinal book. In 2015 Jeon et al. reported that Illite could be used to treat Alzheimer's disease [16].

Zein is a corn protein encompassing ~80% of the protein present in the corn grain. It is a "green" and renewable substitute for petroleum-based or animal-derived polymers in biomedicals. Due to its plant origin, it is rendered non-immunogenic compared to the other protein polymers obtained from animal sources [17]. Among all the plant-based proteins, Zein, a corn protein rich in glutamine, leucine, alanine, and proline, has shown potential as a biomaterial for bone regeneration. Zein improves cell adhesion by interacting with the tissue transglutaminase, an extracellular enzyme [18]. However, Zein offers many advantages, including biocompatibility, biodegradation, adequate strength, low immunogenicity; however it lacks bioactivity [19].

Many polyesters have been studied for tissue engineering applications in the past decade due to their biocompatibility, bioresorbable, and high mechanical strength [20]–[22]. Among them, Polycaprolactone (PCL) is of great interest in tissue engineering. PCL is an aliphatic polyester with a low glass transition temperature (-62ºC) and a melting point ranging between (55—60ºC). Apart from being biocompatible and biodegradable, PCL qualifies for tissue engineering applications due to its ease of availability, low cost, ease of processing, and modification. PCL's biological and mechanical characteristics make it an excellent candidate for musculoskeletal tissue engineering applications [23]–[25]. However, some of the drawbacks of the PCL include its slow *in vivo* degradation and lack of bioactive nature, including hydrophobicity which hinders cell proliferation and adhesion [26]–[28].

To over these drawbacks of PCL, blending PCL with a suitable natural organic/inorganic bioactive component is a convenient way to impart some hydrophilicity and improve degradation rate without sacrificing the biocompatible nature of the polymer. For example, acidic degradation byproducts of PCL and the degradation rate of PCL were both enhanced by blending with chitosan [29]. Blending PCL with polysaccharides like starch, dextran, and gellan can improve the cell adhesion and proliferation of the composite scaffolds [30]. Wu *et al.* prepared Zein/PCL blend fibrous scaffold and reported improvement in hydrophilicity and degradation rate was certainly improved [31]. Dong et al. reported excellent toughness and flexibility of PCL/zein films [32]. Liao *et al.* developed Zein/PCL from two nozzle electrospinning setups. They said that adding calcium lactate to the composite scaffold improves *in vitro* mineralization, cell adhesion, spread, and

proliferation [33]. He et al. developed coaxial core/shell PCL/Zein nanofibers. They achieved the controlled release of metronidazole and reduced toxicity because of the controlled release [34]. Awasthi *et al.* fabricate albumin exfoliated MoS_2 incorporated PCL/Zein nanofibrous scaffolds. They reported increased tensile properties, cell adhesion, and osteoblastic differentiation. In contrast, the degradation rate was slowed done due to the incorporation of MoS_2 [28].

The current study focuses on the fabrication of different concentrations of illite clay incorporated PCL/Zein electrospun nanofibrous scaffolds for the very first time. The composite nanofibrous scaffolds were investigated for relevant physicochemical and biological properties. The authors focused on understanding the effects of illite clay loading on the PCL/Zein fibrous scaffold to improve the physicochemical and biological properties relevant to bone regeneration. The composite scaffolds were evaluated for surface morphology, elemental analysis, mechanical properties, surface wettability, thermal stability, degradation, biomineralization, and *in-vitro* biocompatibility with the MC3T3-e1 cells for their proliferation, cell adhesion, toxicity, and differentiation activity. To the best of the author's knowledge to date, there is no work reported relating to the incorporation of illite clay in any polymeric system for bone tissue engineering purposes. This work would be the first of its kind about the loading of illite clay in PCL/Zein electrospun nanofibers.

4.2 Experimental

4.2.1 Materials

For this study, Zein and polycaprolactone (PCL, average Mn = 80000) were procured from Sigma-Aldrich (USA). Illite clay powder, which was mined in Yeongdong of Chungbuk province, in the Republic of Korea, and acetic acid (>99.7%) purity was purchased from Fujifilm Wako Chemical Ltd. (Japan). All materials, chemicals, and solvents were of analytical grade and used without any further processing.

4.2.2 Fabrication of PCL/Zein (CC) and PCL/Zein/Illite (CCC) composite nanofibers

All electrospinning solutions were prepared in acetic acid as solvent. 20 wt% Zein and 20 wt% PCL were dissolved separately in acetic acid and stirred overnight. For developing PCL/Zein (CC) nanofibers, the PCL and Zein solutions were mixed in a 5:5 ratio. For PCL/Zein/Illite (CCC) nanofibers, different amount of illite clay (2%, 4%, and 6% by weight concerning the PCL/Zein content was first added to the zein solution and stirred overnight. The as-prepared Zein/Illite

solution was then transferred to an equal amount of PCL solution and stirred for another 24h to ensure complete dissolution and homogeneity before moving to the electrospinning device. The produced nanofiber mats were labeled as CC, CCC2, CCC4, and CCC6.

A horizontal electrospinning setup was employed for nanofiber production using a high voltage power supply (Har-100812, Matsusada Co., Tokyo Japan) and a syringe pump (KDS-100, KD Scientific, USA). The spinning solutions were put in a 25 ml syringe attached to a 20-gauge needle and were mounted onto the syringe pump. The pump dispensed the spinning solution at a constant rate of 1 ml/h under a high voltage of 15 kV applied across the needle tip and the drum collector covered with butter paper. The collector to the needle distance was maintained at 150 mm. Electrospinning was carried out at the ambient temperature of 23°C and relative humidity of ~40%. All samples were spun for 20 hours to ensure a uniform thickness of the nanofibrous mats.

4.2.3 Characterization

4.2.3.1 Physicochemical analysis

The surface morphology of the produced composite nanofibers was studied with scanning electron microscopy (SEM, JSM-6010LA, JEOL, Japan) and transmission electron microscopy (TEM, JEOL 2010 Fas TEM, Japan). Before SEM imagery, the samples were sputter-coated with the platinum surface on the carbon tape. Fiber diameters and distribution were measured using Image J software (version 1.4.3). The composite clay nanofibers' elemental compositions were determined with SEM using energy dispersive X-ray (EDX) spectroscopy.

A Fourier transform infra-red (FTIR) spectroscopy (Prestige-21, Shimadzu, Japan) was used to determine the chemical composition of the composite nanofibers, and X-ray diffractometry (XRD, Miniflex 300, Rigaku Co., Ltd., Japan) was done to determine the crystal structure of the nanofibers operating at 30kV and 500 mA. Diffractions were detected in the $2\theta = 5^{o}$—80° range with a step of 0.05^{o}.

Thermal degradability of the composite nanofibers was evaluated by thermogravimetric analysis (TGA, TG-8120, Rigaku Corporation, Japan) with a heating rate of 10°C/min. Thermal degradability was observed within the temperature range 30°C – 500°C under an air atmosphere.

The mechanical performance of the produced fibrous mats was analyzed by subjecting them to tensile tests using the universal tensile testing machine (UTM), Tensilon RTC 250A, A&D Company Ltd., Japan. Stress, strain, and elastic modulus were determined from the obtained UTM data.

The surface wettability of the composite nanofibers was analyzed by water contact angle (WCA) using Digidrop, GBX, France, contact angle analyzer. A static water contact angle was calculated using a 28 gauge needle. Five readings for each type of nanofibers were calculated, and averages were recorded.

Electrical conductivity and viscosity of the polymeric electrospinning solutions were measured by the Horiba D54 handheld pH/conductivity meter and Brookfield viscometer (DV-I Prime).

4.2.3.2 Biodegradation test

Polymeric scaffold degradation is required for the regeneration of a "natural" tissue implant and is followed by the four steps (i) hydration, (ii) loss of tensile strength, (iii) loss of mass, and (iv) solubilization. It is crucial for the scaffolds used as the extracellular matrix in tissue engineering to be dissolved by the surrounding tissues to avoid additional surgeries after the intended purpose [35]. The biodegradation of the CC and clay-loaded nanofibers (CCC2, CCC4, and CCC6 was carried by immersion in 1× phosphate buffer saline (PBS) solution at pH=7.4 and incubation at 37°C for 21 days. The PBS was changed every second day. At the end of the pre-determined time interval, the samples were taken out from the PBS solution, washed with deionized water, and dried at 37°C before calculating for ant mass change in the samples. The degradation was analyzed by the mass loss and the SEM microscopy at the 21-day time interval. The weight loss was determined by the following equation:

$$Weight\ loss\ \% = \frac{W0 - W1}{W0} \times 100 \quad (1)$$

Where W_0 is the initial weight of the samples, W_d is the weight of the samples being incubated in PBS.

4.2.3.3 Biomineralization test

To analyze the role of illite clay in the CC composite nanofibers for the hard tissue engineering, and in vitro mineralization test was performed to investigate the deposition of calcium and inorganic phosphate on the surface of the composite CC and illite clay loaded CC nanofibers after

incubation in the simulated body fluid (SBF). SBF was prepared according to Kokubu's recipe having ion concentrations nearly equal to those of the human blood plasma. The SBF solution of pH=7.4 was prepared according to the given recipe [36] in a 2-liter plastic bottle using deionized water. The manufactured composite nanofibers were cut into 4×4 $cm^{2,}$ placed in the Petri dishes and filled with 20 ml of the SBF solution, and incubated at 37ºC for the desired time intervals (7 and 14 days). During the incubation period, the SBF solution was replenished with the fresh solution every day. At 7 and 14 days, the samples were taken out of the SBF solution, washed with distilled water, and then dried at 37ºC before subjecting to the SEM micrography and EDX analysis. The deposition of apatite was also analyzed by the XRD diffractograms of the incubated samples.

4.2.3.4 Alkaline Phosphatase staining

The ALP expression was determined by the ALP staining kit (Red) (ab242286: Abcam Germany). Briefly, nanofiber samples were sterilized using ethanol 75% for 20 minutes. The samples were washed via 1× PBS thrice for 5 min each. MC3T3-e1 cells were seeded at 10^4 cells/cm^2 on the surface of fiber samples in 96 well multiwall plates using Alpha modified Eagle's minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) with 50 mg/ml ascorbic acid for 7, and 14 days. The medium was replenished on the alternative days. On the pre-determined days, samples were taken out, and cells were fixed with 4% ice-cooled paraformaldehyde and washed with PBS. 0.5 ml of ALP staining solution was added to the samples and incubated for another 30 min at 37ºC, washed with PBS, and images were taken using Olympus optical microscope with DP71 digital microscope camera. Quantification of the active ALP areas was evaluated by ImageJ software (version 1.4.3).

4.2.3.5 Biocompatibility and cytotoxicity assessment

On days 1, 3, and 7 after seeding, the metabolic activity of the MC3T3-e1 cells in the presence of the composite nanofibrous scaffold was determined by the precipitation of the tetrazolium salt (WST-1), as reported earlier [37]. Before the experiment, the prepared nanofiber samples (5 mm discs) were UV irradiated and sterilized with 70% ethanol solution for 1 h and rinsed with PBS afterward. The sterilized samples were placed in the 96 well glass culture plate. MC3T3-e1 cells were cultured in α-MEM containing 10% FBS in a humidified incubator at 37ºC having a 5% CO_2 atmosphere. The wells containing the samples were seeded at a density of 3×10^3 MC3T3-e1 cells per well. The cells were cultured for 7 days. At the pre-determined time intervals 1, 3, and 7 days the cell proliferation was assessed by adding 10 ul WST-1 to each well and incubated for 2 h. The

metabolically active MC3T3-e1 cell will react with WST-1 reagent and produce formazan dye which can be quantified at 450 nm using a microplate reader (Thermos scientific, Multiscan FC Instrument).

The cytotoxic potential of the composite nanofibers on the MC3T3-e1 cells was evaluated using in vitro toxicology assay kit lactate dehydrogenase-based (LDH assay) kit according to the manufacturer's protocol. Briefly, the nanofiber mats were washed with 70% ethanol and then UV irradiated. MC3T3-e1 cells were cultured on the nanofiber scaffolds at a seeding density of 3×10^3 cells/cm^2. The culture media was harvested 24h post-seeding and mixed with the solutions provided with the LDH assay kit. After incubation for 20 min at room temperature and darkness, the lactate dehydrogenase activity was measured using the optical density of the reaction mixture at 490 nm.

Scanning electron microscopy was employed to assess the MC3T3-e1 cells adhesion and morphology on the composite nanofibrous scaffolds. Briefly, MC3T3-e1 cells were seeded on the composite scaffolds. After 72h, the cells were fixed on the scaffolds using 4% paraformaldehyde for 4 h at 4°C. The scaffolds were dehydrated using ethanol solutions with increasing concentration. The scaffolds were vacuum dried and sputter-coated with platinum before subjecting to the SEM micrography for cell adhesion and morphology.

The samples were also evaluated for calcium deposition after seeding scaffolds with the MC3T3-e1 cells using EDX mapping, representing the compositional map of the samples.

4.3 Results and Discussion

4.3.1 Surface morphology and elemental analysis

Surface morphology of neat PCL/Zein (CC) and Illite clay-loaded PCL/Zein (CCC2, CCC4, and CCC6) composite nanofibers are shown in Fig. 4.1. The neat CC nanofibers Fig. 4.1a exhibit bead-free, smooth morphology produced by mixing an equal amount of 20 wt% of PCL and Zein solution. The introduction of illite clay in the PCL/Zein solution resulted in decreased diameters of the produced composite nanofibers. The fiber diameter decreased with increasing the clay concentration. The composite nanofibers (CCC2, CCC4, and CCC6) showed average diameters of 685 ± 314, 482 ± 275, and 335 ± 143 nm, while neat CC nanofibers showed an average diameter of 885 ± 474 nm. The histograms in Fig. 4.1 (a', b', c', and d') shows the diameter variations in the produced neat and composite nanofiber sheets, respectively. The decrease in the fiber diameter can result from the reduction of the viscosity and increase in the electrical conductivity of the polymeric

electrospinning solution. The details of the viscosity and electrical properties of the electrospinning solution are given in Table 4.1. It was reported that increased electrical conductivity and reduction in viscosity improved the spinnability and induced reduction in fiber diameter during electrospinning [38], [39].

Table 4-1 The conductivity and viscosity of the various polymeric and clay electrospinning solution.

Solution	Illite % (w/v)	Conductivity (µS/cm)	Viscosity (cP)
CC	0	0.31±0.01	980.24±3.36
CCC2	2	1.73±0.02	903.42±9.21
CCC4	4	2.31±0.01	872.33±4.98
CCC6	6	4.24±0.01	830.27±7.47

The nanofiber sheets with 0 wt%, 2 wt%, and 4 wt% resulted in bead-free smooth morphology. When the clay content was high 6 wt%, beaded segments appeared. At high clay contents, the dispersibility of illite clay in the electrospinning solution was much difficult.

To determine the elemental composition of the produced nanofiber scaffolds, EDX spectroscopy was employed. EDX spectra of the produced nanofibers are given in Fig. 4.1 (a", b", c", and d"), and the elemental percentages are shown in Table 4.2. The presence of the illite clay in the nanofibrous scaffold was justified by the presence of the elemental Silicon (Si) and Aluminum (Al). As the amount of illite clay content in the composite nanofibers increased, Si and Al also increased, amounts given in Table 4.2.

Table 4-2 Elemental analysis of the fabricated nanofibrous mats.

	C	N	O	**Si**	**Al**	Fe	Na	Mg	K	S	Ca	Ti
Sample	w%	w%	w%	**w%**	**w%**	w%	w%	w%	w%	w%	w%	w%
CC	57.27	16.93	25.80	**----**	**----**	----	----	----	----	----	----	----
CCC2	62.32	14.43	19.50	**0.87**	**0.26**	0.39	----	0.03	0.19	1.96	0.04	0.01

CCC4	61.34	12.16	22.41	**1.34**	**0.41**	0.39	0.39	0.03	0.28	1.21	0.03	0.01
CCC6	61.17	14.60	18.87	**2.17**	**0.53**	0.65	0.44	0.03	0.33	1.16	0.04	0.01

Figure 4-1 SEM micrographs of (a) CC, (b) CCC2, (c) CCC4, and (d) CCC6 electrospun nanofibrous scaffolds. (a'– d') the histograms for fiber diameter distribution. (a"—d") EDX elemental analysis of the CC and CCC scaffolds.

The incorporation of the illite clay in the composite nanofibers was confirmed by the TEM images Fig. 4.2. The TEM micrographs show that the illite clay is not evenly dispersed in the electrospun nanofiber but forms agglomerates. This could be due to the organophobic nature of the clays. The agglomerates became bigger and bigger as the illite clay content in the electrospinning solution increased, thus results in the bead formation in the nanofibers as confirmed by the SEM micrographs in Fig. 4.1d.

Figure 4-2 TEM images of (a) CCC2, (b) CCC4, and (c) CCC6.

4.3.2 FTIR fingerprints

FTIR fingerprints of the Illite, Zein, PCL and the composite nanofibrous scaffolds are given in Fig. 4.3a. The characteristic peaks of the illite clay appeared in the region of 1250 and 750 cm^{-1} [40]. Illite is an inorganic compound mainly composed of Al2O3 and SiO2; most of the bands in the FTIR spectra are associated with —Si —O— and —Al—O— [41]. The bending and stretching of Si—O were observed at 1089 cm^{-1} and 1039 cm^{-1}. The –OH bending vibrations in Al—OH—Al was seen at 922 cm^{-1} [42]. The peaks at 791 cm^{-1} and 801 cm^{-1} were noted as the compounded vibrations of the Si—O-- Al^{iv} [43]. The peak at 3624 cm^{-1} was due to the –OH stretching vibrations in the metallic layers and adsorbed water [44].

The pristine zein FTIR spectra show broad bands around 3322 cm^{-1} and 3201 cm^{-1} due to the amide A (N—H) stretching and vibration contributions. The absorption peaks between 3000 cm^{-1} and 2800 cm^{-1} are due to the —C—H stretching in the aliphatic groups. The characteristics of protein peaks at 1660 cm^{-1} (amide I), and 1538 cm^{-1} (amide II), were indicative of the –C=O stretching and vibrations and stretching of the C—N bonds. The bands at 1455 cm^{-1}, 1317 cm^{-1}, and 1255 cm^{-1} are due to a combination of N—H and C—N stretching and bending and due to the C—H deformation of the methyl group [45]–[47].

The pristine PCL shows a characteristic carbonyl peak at the 1722 cm^{-1} (C=O stretching) [48]. The stretching vibrations of the CH_2 groups were observed at 2951 cm^{-1} and 2870 cm^{-1}. The bands are associated with the crystalline phase at 1294 cm^{-1} and the amorphous phase of the C—O—C group at 1166 cm^{-1} [49]. The absorption peaks at 1473 cm^{-1} (asymmetric deformation of CH_3), 1367 cm^{-1} (symmetric wagging of CH_3), 1242 cm-1 (CH_3 vibrations), and 1042 cm^{-1} (C—O stretching and CH bending) were observed [50].

The FTIR fingerprints of the pristine Zein and PCL are all present in the composite CC nanofibers. Briefly, the CH_2 stretching modes were found at 2951 cm^{-1} and 2870 cm^{-1}. The PCLs carbonyl peak was observed around ~1730 cm^{-1}, the bands around 1665 cm^{-1}(amide I) and 1525 cm^{-1}(amide II) were associated with the proteins in the Zein. The absorption peaks between 1500 cm^{-1} and 1000 cm^{-1} were associated with the stretching and vibration of the N—H, C—N, and C—H deformation in Zein and CH_3 asymmetric, symmetric deformation, wagging, and deformation. All the peaks observed in the CC nanofibers were also present in the Illite clay-loaded CCC nanofibers. The change in the intensity of the absorption peaks confirms the presence of Illite in the nanofibrous scaffolds.

4.3.3 XRD

The XRD diffractograms of the pristine materials and the composite nanofibers are shown in Fig. 4.3b. The neat Zein records two broad peaks (amorphous form) at around 2θ of 9° and 20°, attributed to the α-helix structure of the zein protein [51], [52]. The pristine PCL showed the inherent peaks at 2θ of 21°, 24°, and 31°, which are assigned to the semicrystalline structure of the PCL (1999 JCPDS file no. 96-720-5590) [53]. In the composite PCL/zein nanofiber (CC), the broad diffraction peak of the Zein at around 17° to 25° is dominated by the semicrystalline nature of PCL polymer. In pristine illite clay XRD diffractograms, the peaks at 2θ of 8.7° and 2θ of 26.8° represent the corresponding (001) and (003) crystal planes existing in the Illite with the basal spacing of 10 Å and 3.34 Å [54]–[56]. After adding illite clay in the composite CC nanofibers, additional peaks are exhibited in the CCC composite nanofibers at 2θ = 8.7° and 2θ = 26.8°, corresponding to the illite crystal planes of (001) and (003).

4.3.4 TGA

The thermal behavior of the CC and CCC nanofibers was evaluated by TGA, as shown in Fig. 4.3c. The CC and CCC composite nanofibers showed a three-stage weight loss for all compositions. The first stage (25ºC to 100ºC) is associated with the evaporation of the surface adsorbed water resulting in a weight loss of 3.63% for CC, 3.86% for CCC2, 3.79% for CCC4, and 4.24% for CCC6. The addition of Illite has minutely improved the water absorption ability of the composite nanofibrous scaffolds. The second stage associated with the molecular chain fracture and thermal decomposition of Zein started above 290ºC. The third stage, above 370ºC associated with the thermal degradation of the PCL [57]. There was no significant difference between the decomposition temperatures in the CC and CCC composite nanofibers. However, CCC composite nanofibers showed a slight difference in remaining weight percentage compared to CC nanofibers, as shown in the inset of Fig. 3c. After complete thermal decomposition of the scaffold, thermal stability was obtained from 440ºC to 500ºC and a lower weight loss percentage of 75.1% for CCC6, 81.27% for CCC4, 81.22% for CCC2 as compared to 84.63% for CC nanofibrous scaffold.

4.3.5 Water Contact Angle

The surface wettability characteristic of CC and CCC electrospun composite nanofibrous scaffold with different concentrations of illite clay (2, 4, and 6%) was determined by a static water contact angle analysis. The WCA was determined at water droplet residence time intervals of 1 sec, 2 sec, and 3 sec, as shown in Fig. 4.4a. The CC nanofiber scaffolds showed a WCA of 66.98º at 3 sec of resident time. The WCA constantly decreases upon adding illite clay (26.76º for CCC2, 26.52º for CCC4, and 17.2º for CCC6), as shown in Fig. 4.4b. The enhancement in the hydrophilicity of the composite CCC nanofibrous scaffolds can be due to the inherent hydrophilic nature of the illite clay containing metal oxides and the hydroxyl groups in its composition [58]. Similar experimental results have been reported previously upon the interaction of clay fillers in PCL-based nanofibers [59], [60]. Surface with strong hydrophilicity has been reported to have reduced bacterial adhesion [61], thus indicating to potential to be used in biomedical setups.

Figure 4-3 (a) FTIR. (b) XRD, and (c) TGA of the respective electrospun CC and CCC nanofibrous scaffolds.

4.3.6 Mechanical properties

For hard tissue engineering, the mechanical properties of the scaffolds are among the critical paramount. The stress/strain curves, Young's modulus, the tensile strength of the CC, and different amounts of Illite clay-loaded CCC composite nanofibers were studied as shown in Fig. 4.4 (c) and (d). The different amounts of clay have affected the mechanical properties of the composite nanofiber mats. The CC nanofibers showed the highest strain percentage of ~121%. On the contrary, the clay-loaded CCC composite nanofibers had lower subsistence to distortion. CCC6 nanofibers showed the highest tensile strength of ~6.87 MPa of all other nanofibers scaffolds. As the illite clay increases from 2 to 6 wt%, the tensile strength and Young's modulus of the composite nanofibrous scaffolds improve. This increase could be attributed to the van der Waal attractions between the hydroxyl groups in the illite clay and the functional groups present in PCL and Zein. A similar trend has been shown in a previously reported study by Liao *et al*. They noted that adding calcium lactate in PCL/Zein nanofibers increased the mechanical strength and Young's modulus [33].

4.3.7 Degradation study

The weight loss percentage and morphological changes that occur during 21 days of incubation in 1× PBS solution are shown in Fig. 4.5. Bone regeneration is a fast process and usually take 2 to 3 months to regenerate minor bone fracture and defects fully. So for bone tissue engineering, an ideal scaffold should have a matching degradation rate to the bone repair process. Pure PCL scaffolds usually take almost 2 years to degrade *in vivo* [62]. So incorporating polymers and other bioactive agents can enhance the degradation rate of PCL-based scaffolds to match the bone repair process. The results showed that the CC nanofibrous scaffold showed a weight loss of ~30.55 % after 21 days of incubation. However, the degradation of the PCL is slowly followed by the hydrolytic cleavage of the ester groups either by surface or bulk degradation. This increased weight loss of the CC nanofibrous scaffolds can be due to rapid degradation associated with the Zein [63]. Furthermore, CCC (2, 4, and 6%) composite nanofibers showed even more significant weight loss of 34.28% for CCC2, 37.23% for CCC4, and 44.31% for CCC6, respectively. The incorporation of illite clay has improved the degradation rate. This may be due to the smaller fiber diameter and enhanced hydrophilicity of the CCC scaffolds compared to the CC scaffold, which accounts for an increase in the diffusion of PBS within the scaffolds and promotes hydrolytic degradation.

Figure 4-4 Surface wettability (a, b) of the CC and CCC scaffolds with WCA imagery. Mechanical performance, (a) stress/strain curves, and (b) histograms showing the tensile strength and Young's modulus of different as-spun composite scaffolds.

SEM micrographs in Fig. 4.5b show that after 21 days of incubation, the CC scaffold does not show any significant structural deformation. The minor fiber distortions could be accounted for the chain disorder due to the zein degradation. In general, CC scaffolds showed surface erosion degradation. CCC scaffolds showed a distinct degradation behavior. The addition of clay has resulted in the breakage of the fibers. The thinner fibers in the scaffolds were severely damaged—the damage in scaffold increase as the amount of clay in the scaffolds increases. The breakage in the thicker fibers in the CCC scaffolds indicates a bulk degradation phenomenon. Similar results have been reported previously by Gaharwar *et al*., where they studied the degradation of PCL nanofibrous scaffolds loaded with synthetic nanoclay (Nanofil® 116) [26]. Overall, the incorporation of illite clay has enhanced the composite scaffolds' degradation and facilitated the bulk degradation of the scaffolds.

Figure 4-5 Degradation assay, (a) histograms for weight loss percentage, and (b) SEM micrographs for the respective scaffolds after 21 days of incubation.

4.3.8 *In Vitro* Biomineralization

The bone-bonding characteristics of the artificial biomaterials are a prerequisite for the development of bone tissue engineering scaffolds. This ability of the materials is often evaluated by the formation of an apatite-like structure on its surface when coming in contact with SBF with an ionic concentration nearly equal to the human blood plasma [64]. The *in vivo* bioactivity of the scaffolds is highly dependent on the formation of apatite depots formed on the material's surface when immersed in SBF. This *in vitro* assay provides an indirect insight into the bone-bonding ability of the newly developed biomaterials.

Fig. 4.6 shows the apatite mineral deposition on the CC and CCC nanofibrous scaffolds. The deposition of the mineral was evaluated as the Ca/P ratio given by the EDX. It can be observed from the SEM micrographs that the apatite clusters seem to be denser on the surface of the CCC scaffolds rather than the CC scaffold at days 7 and 14. The factors for the higher apatite nucleation on the CCC scaffolds could be the surface exposed clay providing sites for the apatite nucleation [8]. The other factor could be the surface roughness formed on the nanofibers due to the incorporation of the clay, as surface roughness facilitates the nucleation process, especially in the saturated ionic solution like SBF [26]. PCL carbonyl group hydrolysis and form –OH and –COOH groups in SBF solution dissociate into carboxylate anions that combine with Calcium and phosphate ions and enhance the apatite nucleation process.

Furthermore, the Ca/P ratio on the scaffolds was also evaluated by the EDX and is shown in Fig. 4.6 (i, j, k, and l) and Table 4.3. The Ca/P ratio for the CC scaffold after 14 days of incubation in SBF was calculated to be 1.25, whereas the Ca/P ratio for the CCC scaffolds was 1.41 for CCC2, 1.45 for CCC4, and 1.55 for CCC6. The XRD diffractograms of CC and CCC composite scaffolds after incubation in SBF for 14 days show new reflection at 2θ of ~32°, the characteristics of the hydroxyapatite, thus confirming the formation of mineral apatite on the fiber surface [65]. The obtained results suggest that the CCC composite scaffolds are a promising material for bone cell regeneration.

Figure 4-6 SEM images for apatite surface mineralization from (a—d) 7 days, and (e—h) 14 days, (i—l) respective EDX spectra's, (m—p) XRD of the respective scaffolds after mineralization.

Table 4-3 Elemental analysis of the composite nanofiber mats after biomineralization.

Element	CC	CCC2	CCC4	CCC6
	w%	w%	w%	w%
C	53.42	56.78	57.26	51.73
N	11.50	10.60	7.54	6.33
O	20.68	18.27	20.14	13.15
Na	3.41	3.31	3.01	7.87
Mg	0.02	0.01	0.01	0.01
P	**2.75**	**2.57**	**2.79**	**3.34**
Cl	4.59	4.31	4.60	11.80
K	0.20	0.08	0.13	0.24
Ca	**3.43**	**3.62**	**4.04**	**5.18**
Si	----	0.37	0.39	0.29
Al	----	0.08	0.09	0.06

4.3.9 Alkaline phosphatase expression

The effect of illite clay incorporation in the CCC nanofibrous scaffolds on the osteogenic differentiation of MC3T3-e1 cells was investigated by incubating MC3T3-e1 cells on CC and CCC composite scaffolds. The osteogenic differentiation was determined by ALP staining at 7-day and 14-day time intervals. The ALP active area was quantified by ImageJ software. It was found that on 7 days, the CC nanofibrous scaffold showed the minimum ALP active area (bright pink color), as shown in Fig. 4.7. CCC nanofibrous scaffolds showed a higher ALP active area. On 14 days, all developed scaffolds showed an improved ALP activity of above 50%. CCC6 scaffold showed the highest ALP active area (~84 %). The ALP active area is reduced as the amount of clay in the scaffold reduces. The reason being that Illite is an aluminosilicate and is hence predicted to be

osteoconductive, as silica is an osteogenic agent [66]. The higher the ALP activity will result in rigorous bone-like protein deposition, which will further help in bone mineralization.

Figure 4-7 Effect of Illite clay incorporation on the osteogenic differentiation of MC3T3-e1 cells, (a) MC3T3-e1 cells stained for ALP activity after 7 and 14 days. (b) Histograms for quantitative assessment for ALP activity of the respective scaffolds.

4.3.10 In vitro cell viability and cytotoxicity with MC3T3-e1 pre-osteoblast cell line

The WST-1 spectrophotometric assay quantitatively determined MC3T3-e1 cells viability on the CC and CCC composite scaffolds. The absorbance values were noted at pre-determined time intervals of 1, 3, and 7 days. The WST-1 assay results (Fig. 4.8a) show that the different CCC composite scaffolds showed a higher absorbance value than the CC scaffold, especially at a more extended incubation period (3 and 7 days). The continuously increasing absorbance in the CCC scaffolds at different time intervals indicates improved cell proliferation. The results imply that the illite clay incorporation has enhanced the biocompatibility and cell viability of the composite CCC scaffolds.

The LDH release assay assessed cellular death or the toxicity of the CC and CCC scaffolds. The assay is associated with the release of the LDH enzyme from the damaged plasma membranes in the cellular culture. The higher the damage, the more will be LDH release, and the greater will be cytotoxicity. The spectroscopic quantification is given in Fig. 4.8b. The results show no significant difference in the LDH release between the two sets of nanofibrous scaffolds CC and CCC.

The cell adhesion on the surface of the scaffold is a crucial consideration for tissue engineering applications. So, SEM images were taken to evaluate the cell interaction with the different scaffolds at 7 days of incubation. All samples showed good cell adhesion, as shown in Fig. 4.8c. Overall, CCC scaffolds showed better spreading of the MC3T3-e1 cell over the scaffold surface. This enhanced cell proliferation could be due to the better biocompatibility and wettability of the CCC scaffolds as compared to the CC scaffold.

Mineralization is also an important osteogenic differentiation marker and was investigated on day 3 and 7 of MC3T3-e1 cell culture with the CC and CCC scaffolds. An increasing trend of calcium deposition was observed and can be seen in the EDX elemental mappings for calcium in Fig. 4.8d. Furthermore, the calcium deposition increased as the number of cell culture days increased, and as the amount of clay content increased, the calcium deposition increased. The ALP activity results support the results here.

Figure 4-8 In vitro biocompatibility, (a) WST-1 assay results for the MC3T3-e1 cell proliferation, (b) LDH release assay for cellular death/toxicity, (c) SEM micrographs for the cell adhesion and migration, and (d) EDX mapping for the calcium mineralization after MC3T3-e1 cell culturing for 3 and 7 days.

4.4 Conclusion

In this study, we report on the physicochemical and biological characterization of the as-spun clay composite scaffolds. A range of nanofibrous scaffolds with varying amounts of illite clay was fabricated. Illite addition resulted in nanofiber diameter reduction but was also plagued with non-uniformity and beads formation. The FTIR, EDX, and XRD diffractogram confirmed the incorporation of illite clay in the PCL/zein nanofibers. Illite clay enhanced the surface wettability and in vitro biomineralization (Ca/P ratio) of the CCC scaffolds.

Furthermore, clay addition improved the mechanical performance of the scaffolds, which is vital for hard tissue engineering applications. The WST-1 and LDH assay confirmed that the clay composite scaffolds had enhanced biocompatibility and cell viability when incubated with the MC3T3-e1 cells. The enhanced wettability of the scaffolds also resulted in improved cell adhesion and proliferation on CCC scaffolds compared to CC scaffolds. In addition, the CCC scaffolds showed improved cell differentiation and mineralization, as shown by ALP staining assay and EDX mapping. Overall, the results indicate that the illite clay incorporated scaffolds can serve as a promising material for musculoskeletal tissue engineering.

Based on physicochemical and *in vitro* biological assessment, the clay-corn-caprolactone composite scaffolds show promising results for their usage in hard tissue engineering applications. The prospect will be to promulgate the efficacy of the clay-loaded scaffolds by undergoing detailed *in vivo* analysis.

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Chapter 5

Conclusion

This dissertation emphasis on the electrospun nanofibrous scaffolds containing naturally sourced bioactive materials for regenerative medicine. Despite all the advances made over the past few decades in developing wound dressing, wound healing remains a challenge for medical practitioners. To mimic all the properties of natural and health skin and tissue remain a challenge for the engineers. In this scenario electrospinning has gained a lot of attention in regenerative medicine because of it excellent ability to fabricate nanostructures able to correspond to natural extracellular matrix which can support and hold the cellular matrix and allow it to grow thus improving the healing rates. The nanostructure of the fibrous scaffold further improves the regeneration rate by providing the bioactive ingredients to the repair site directly. The three naturally sourced ingredients used in this study were Manuka honey; known for its bioactive nature due to the presence of the methyl glyoxal (MGO), Blumea balsamifera essential oil; used in Chinese medicine for the cure of many skin related diseases; and illite clay; an aluminosilicate capable to enhance the osteoconductivity of the scaffolds intended for bone tissue engineering.

The first two research works were related to the fabrication of antibacterial, antioxidant, and biocompatible wound dressings. The work utilized cellulose acetate as the polymeric nanofiber carrier. The nanofiber containing bioactive agents; Manuka Honey and Blumea oil were successfully developed on a horizontal electrospinning setup. The nanofibrous mats showed good antibacterial properties. The antioxidant properties were time dependent and were based on the amount of bioactive ingredient present in the nanofibrous mats. The release study showed bi-phasic release profile in case of blumea balsamifera and followed a first order release kinetics. The surface properties of the nanofibrous mats depends on the bioactive ingredients included in them. The Manuka honey loaded mats showed a hydrophilic surface and blumea oil loaded mats showed a hydrophilic property as shown by the water contact angle study. Both nanofibrous mats showed good cell viability when cultured with NIH 3T3 cell line.

The third research work was related to the fabrication of illite clay loaded PCL/Zein nanofibrous scaffold for tissue engineering. The work was focused on the utilization of illite clay as an ingredient to improve the mineralization ability of the composite scaffold, along with enhanced osteoblastic differentiation. The prepared scaffolds were subjected to physicochemical and

biological evaluation. The illite clay loaded nanofibers showed decreased fiber diameters and improved strengths. The scaffolds showed improved biomineralization when subjected to simulated body fluids. The biomineralization was evaluated for calcium to phosphate ratio the scaffold with 6% illite clay showed a Ca/P ratio of 1.55. There was no significant impact of illite clay incorporation on the toxicity of the scaffolds when subjected to LDH assay. The WST-1 assay showed that the clay incorporation has improved the cell viability of the MC3T3-e1 pre-osteoblast cells when cultured with the scaffolds. Alkaline phosphatase assay showed that the osteoblastic differentiation in the illite loaded scaffolds was more than in the pristine PCL/Zein nanofibers.

Based on physicochemical and *in vitro* biological assessment, the fabricated bioactive composite scaffolds show promising results for their usage in regenerative medicine. The prospect will be to promulgate the efficacy of these scaffolds by undergoing detailed *in vivo* analysis.

Accomplishments

(a) Peer-reviewed publications

1. **A. Ullah**, Y. Saito, S. Ullah, M.K. Haider, H. Nawaz, P. Duy-Nam, D. Kharaghani, **I.S. Kim**, Bioactive Sambong oil-loaded electrospun cellulose acetate nanofibers: Preparation, characterization, and in-vitro biocompatibility, Int. J. Biol. Macromol., vol. 166, pp. 1009–1021, January 2021.

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