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Effects Of Electrospun Scaffolds On Epithelial Cell Growth And Morphology

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Biographical Sketch

Divya Shankar Iyer started her academic career at Visvesvaraya Technological University in Bangalore, India in pursuit of a degree in Engineering Biotechnology. Her passion in improving life quality of people around the world using biotechnology led her to develop an interest in nano biotechnology. This brought Divya to join the Joint School of Nanoscience and Nanoengineering in 2013 for her Master's degree in Nanoengineering. She plans to enroll in the doctoral program at JSNN to help formulate understanding of the principles and mechanisms of cells involved at the nano scale. Ultimately, Divya plans to implement her training to create new advancements in nanomedicine.

Divya enjoyed a unique childhood in several countries around the world and is 24 at the time of writing this thesis. She enjoys coupling art with biology. Divya looks forward to the future of nano biotechnology and the vast number of opportunities it holds.

Dedication

This thesis is dedicated in memory of my loving father.

Acknowledgments

First of all, I would like to thank the unending encouragement and guidance from my advisors: Dr. Lifeng Zhang and Dr. Dennis R. LaJeunesse. Without their insights and steadfast support, none of this would have been possible. I also would like to thank the immense support and encouragement of my family as well as my friends at JSNN who have become my adopted family. They have been with me at times of need. When everything seemed bleak and far from possible, they have lead me into the right path. Special thanks will be given to Matthew Kaufmann who has been a constant guiding light from the beginning to the end.

Table of Contents

List of Figures

List of Tables

Abstract

Regeneration of cells on a scaffold is a main aspect in tissue engineering. Nanofibrous scaffolds from electrospinning provide great potential to engineer scaffolds for desired tissue engineering applications because they can mimic in vivo extracellular matrix (ECM). However, there is little known about how the microstructure and surface properties of electrospun nanofibrous scaffold influences the migration and growth of cells. Fundamental studies is demanded to understand the interaction between nanofibrous substrate and cells. In this research, four typical electrospun nanofibrous scaffolds including polyacrylonitrile (PAN), carbon, cellulose acetate (CA) and cellulose were prepared, characterized and then employed as scaffolds for Madin Darby Canine Kidney Epithelial Cells (MDCK). Effects of these electrospun nanofibrous scaffolds on MDCK's growth and morphology were investigated by confocal microscopy. The results from this research provide bioengineers with basic understandings on the mechanisms involved in cell-nanofibrous substrate and cell-cell interactions.

CHAPTER 1

Introduction

1.1 Project objective and significance

The primary objective of this thesis is to characterize the physical properties of four electrospun nanofiber scaffolds and determine how these properties affect the growth and morphology of Epithelial Cell. Specifically the objectives of this research are

I. Surface characterization of electrospun nanofiber scaffold

II. Investigation of the effects of nanofiber scaffolds on the growth and viability of the epithelial cells

III. Investigation of the effects of electrospun scaffolds on epithelial morphology

This work will serve as preliminary and fundamental research that will help us to determine which materials properties of electrospun nanofiber scaffold surface are critical to proper growth, viability, and morphology of a cellular epithelium. The information is important for utilizing these electrospun nanofibrous materials in regenerative medicine for correcting damaged tissue and wound healing.

1.2 Tissue Engineering (TE)

As late as 1987, the field of Tissue engineering introduced the definition that: “Tissue Engineering is the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain, or improve function.” [1]

Tissue Engineering and Regenerative medicine are now considered as central disciples of biomedicine. The three main aspects of TE are: 1. Reprogramming 2. Replacement and 3. Regeneration.

1. Reprogramming is altering the expression of the genetic code. Cellular reprogramming can be achieved using molecular devices, such as: New cells can be generated by rerouting and/or reactivating internal cell differentiation or cell fate pathways through the expression or addition of specific genetic factors or by changing cellular morphology [2-4] This is applicable for drug delivery, nerve synapse bridging, immune system reprogramming, etc.
2. Replacement is exchange of damaged tissue with functional tissue alternatives. When the body is incapable of managing damaged or flawed tissue systems, medicine often prescribes the replacement of these systems. This is often performed using organ and tissue transplants. Synthetic biomaterials provide a new and sometimes novel source for replacement of tissue systems such as bioinert polymers for cartilage replacement [5].
3. Regeneration is the reactivation of primary cellular functions. Physical trauma or damage to tissues may result in a severe compromise to their function; in order to remedy this, a method for generating the missing portion of the system needs to be implemented. The most common form of this remedy is utilizing biomaterials to encourage cellular development in such a manner as to complete the desired tissue system.

Tissue engineering practices oftentimes involve the interaction of tissues with external materials and certain parameters must be accounted for while choosing applicable materials. An ideal material would be biocompatible, biodegradable, immunocomplacent and minimize secondary cellular responses.[6, 7] Better understanding of cell-substrate interactions has enabled

doctors to switch from more traditional materials to more biocompatible and sustainable materials. For instance the change of denture materials from wood to ivory to the growth of teeth from stem cells derived from urine. [8] Titanium and Aluminum have historically been used as biomaterials in hip replacement surgeries [9-11] and the future material for hip replacements may be artificially grown hips.

In this era of personalized medicine, Tissue engineering gains special importance. Using tissue engineering, we can grow replacement organs from our very own cells. [12-14] The same type of genomic information and technology that will help us develop custom designed drugs will also be used to select cells and materials that suit the patients individual; genotypic needs. The fundamental technology today in the application of TE is the growth of cells on various surfaces.

1.3 Tissue Engineering Scaffold and Design

Tissue engineering scaffolds are a 3D matrix used as a substrate for growing cells. [15] The scaffolds and their interactions with cells produce an artificial extracellular matrix (ECM) which plays a pivotal role in accommodating the cells. ECM is a stable structural material that lies under the epithelial. [15] The ECM is in a state of dynamic equilibrium with its environment. The ECM is composed of cell secretions specific to the organ surface. ECM proteins (e.g. Collagen, laminin, fibronectin) secreted by the cells onto the ECM provide the means by which neighboring cells contact and communicate with each other and their surroundings. [16, 17] The cells then undergo proliferation, migration, and differentiation, leading to the formation of a specific tissue while secreting the ECM that is required for tissue regeneration; therefore, designing the scaffold with the maximal attributes of natural ECM is of great importance and a prerequisite for tissue engineering. [18] We hypothesize that the

properties of the electrospun nanofiber scaffold (i.e. the physiochemical surface properties as determined by the composition and structure of the nanofibers) will determine and control the manner in which the cells interact with scaffold and each other.

TE uses cells and scaffolds and combines them to form functional tissue constructs. Scaffolds are used as templates to grow cells. Scaffolds provide the mechanical support on which the cells grow along with replicating key elements of the extracellular matrix (ECM) found naturally occurring in tissue systems and also the important cues for cell viability, morphology and differentiation. The tailoring of scaffolds with such biomimetic and structural properties is an important consideration to be taken into account while choosing a material. Scaffold design is important to influence cellular behavior. The factors governing the design are complex and are often done on a case by case basis depending on the tissue being generated.

Critical scaffold properties are [19] :

1. Sufficient surface area to encourage cell growth and migration, and to allow space for development of vascularization.
2. Pore size and morphology to encourage cell migration into the scaffold and allow high seeding density.
3. Acceptable biocompatible chemical compositions to account for immunocomplacency
4. Bio adsorption enabling the transfer of metabolites
5. Shape/design that facilitates good cellular adhesion and proliferation
6. Mechanical strength and durability to shield cells from biomechanical stresses.

The different types of scaffold also play an important role in influencing cell growth. They can be hydrogels, microparticles or membrane based systems. Of these, the sol-gel

hydrogel and microspheres are injectable forms while the matrix systems are implantable forms. [19]

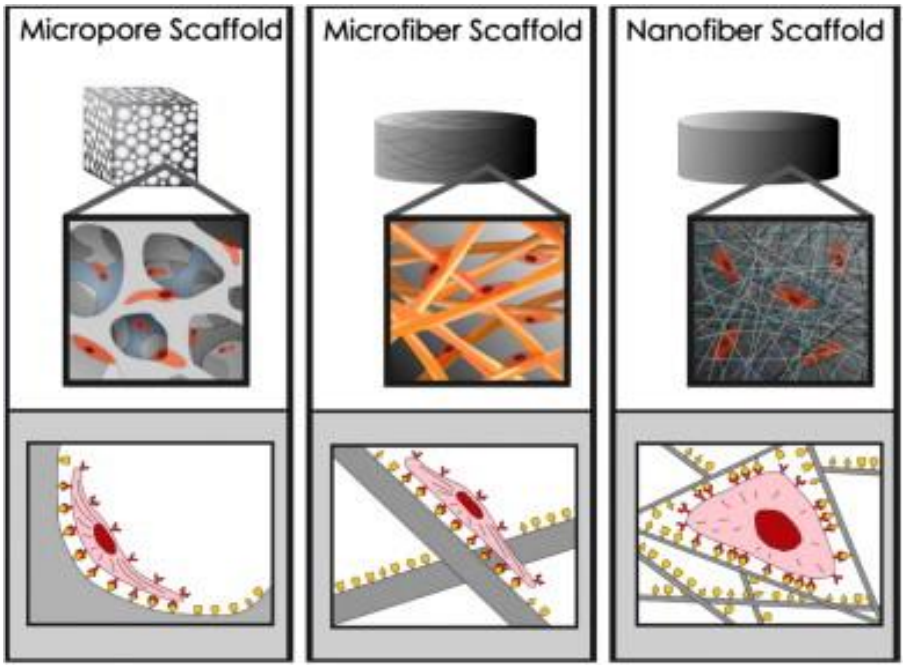


Figure 1-1. Cellular interactions with micro and nano scaled features[20]

Scaffold design is a biomimetic process that involves the generation of synthetic versions of naturally occurring 3D tissue architecture to supports cells growth and/or differentiation from the macro down to the nanoscale. Various methods of scaffold production include fiber mesh, fiber bonding, melt molding, solvent casting/particulate leaching, gas foaming, phase separation, freeze-drying, particle sintering, high-pressure processing and electrospinning. Combinations of these techniques have also been applied in the manufacturing process for scaffolds. [19, 20] Recent studies have shown that nano topologies provide structural cues that are far more similar to the characteristics of natural topologies.

Cells subjected to interaction with microporous and microfibrous interaction behave in a manner similar to their interaction with 2D surfaces. [19, 20] Their interaction with nanofibrous surfaces is completely different. Recent studies have shown that nano topologies provide

structural cues that are far more similar to the characteristics of natural ECM topologies. At the nanoscale, the increased surface area provides a more substantial set of interactions with the cells. Cell-substrate interactions occur on more than just one dimension. These interactions influence the production and secretion of the ECM. They also influence the receptors exhibited by cells and the proteins secreted for adhesion. Nanoscale topologies affect both the cell binding and cell spreading. Interactions with microscale surfaces make the cell have a flat morphology. Cells spread out and flatten themselves to the substrate surface.[21] However, with nanotopologies, these interactions are far more similar to the actual environment inside the body. [22] The cells conform to the nanofibrous topology in different ways. The influence of nanofibrous topologies on cells is worth investigating.

1.4 Motivation for Electrospinning

A technique that has been used to generate such nanotopologies is electrospinning. It is a versatile and economical technique that can generate nanofibers in a very short period of time. Electrospinning is an electrostatically driven method by which micro and nano scaled fibers are formed. A nanoscale topology is acquired by directing an electric current at optimal voltages to ensure fiber size. This method can be optimized to form aligned fibers. In order to control nanofiber size and structure, we can vary and optimize processing parameters including: solution viscosity, voltage, feed rate, solution conductivity, capillary-to-collector distance, and orifice size [12].

Electrospun fibers have a wide range of applications including but not limited to: scaffolds in tissue engineering, biomedical, filtration, wound healing, affinity membrane, protective clothing, energy generation, enzyme immobilization and drug delivery. [23]

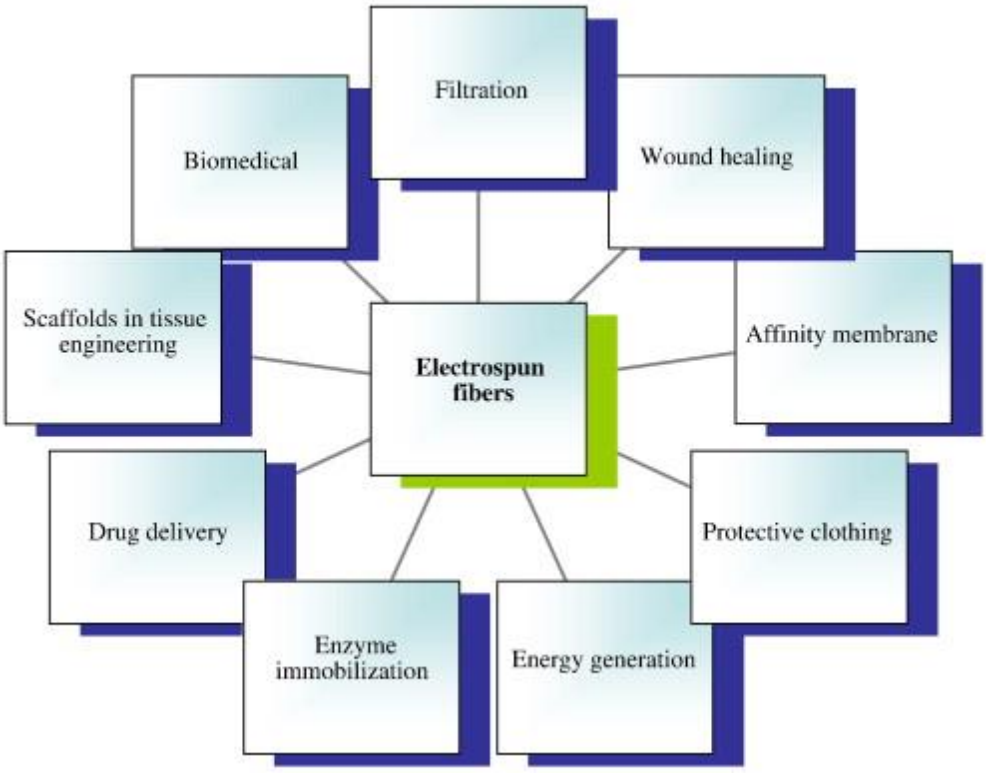


Figure 1-2. Various applications of Electrospun fibers [23]

From a biological standpoint, the ECM and its topographic features can be mimicked using nanofibrous scaffolds. The use of nanotechnology techniques awards the added advantages of high surface area and the ability to provide a designer set of nanoscale features for directing and controlling specific cellular interactions. Almost all tissues and organs are hierarchically organized in a fibrous form, thereby the natural fibrous nature of electrospun membranes serve as features similar to those existing in nature [24]. It is well known that the cells live in a complex mixture of pores, ridges, and fibers of the ECM at the nano level in the body [25].

The scaffolds contain nanofibers with microscale interconnected pores, loose three-dimensional assemble, resembling the topographic features of ECM, and resulting in suitable substrates for tissue engineering [26].

Electrospinning is a simple and inexpensive method of producing nanofibers within a short time frame. It provides the added advantages of material selectivity, determination of thickness, alignment and density of the fibers produced allowing precise tailoring of scaffold properties. Synthetic, natural and hybrid materials can be electrospun for tissue engineering applications [27].

1.5 The Electrospinning Process

A typical electrospinning system is composed of the syringe pump, collector and power supply. (Figure 1.3 [23]). A syringe pump is used to force out a polymer solution through the needle at the very end. A high voltage DC power supply provides the electric charge into the needle.

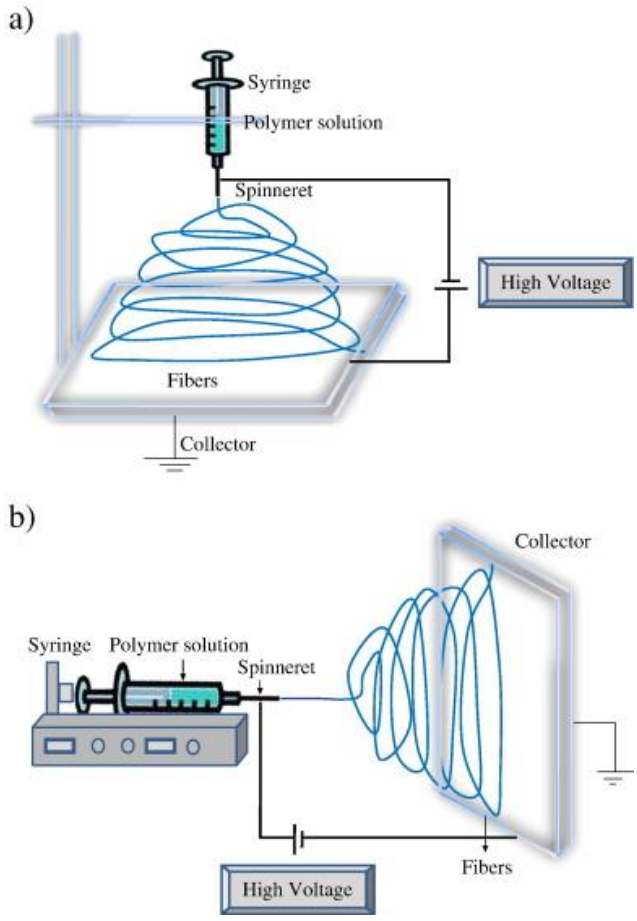


Figure 1-3. Electrospinning setup a) vertical b) horizontal [11].

The accumulated charge forces the polymer solution to emit droplets from the apex of the needle if the electrostatic force of repulsion overcomes the surface tension to form a Taylor cone. If this criteria is fulfilled, the droplets cohesively for fibers that are collected on the collector plate.

1.6 Materials for Scaffolds/ Polymers for Electrospinning

Electrospun materials can be categorized as naturally derived and synthetic polymers or a blend of both including proteins. A wide range of polymers has thus far been electrospun. Table 1.2 shows biodegradable polymers used in scaffold engineering.

Naturally derived polymers have certain advantages over synthetic polymers. These include biodegradability, biocompatibility and low immunogenicity. Natural polymers also have the inherent ability to interact with cells in a smooth manner due to the presence of certain protein sequences like arginine, glycine and aspartic acid. [23, 28] Chitin, chitosan, Collagen, fibronectin and gelatin amongst others, are widely used natural electrospun polymers in tissue engineering. [28-32]

Table 1-1

Table showing natural and synthetic polymers for Electrospinning [25]

Naturally derived	**Synthetic**
Collagen	Poly(lactic acid)
Gelatin	Poly(glycolic acid)
Chitosan	Poly(lactic-co-glycolic acid)
Chitin	Poly(ϵ-caprolactone)
Cellulose	Poly(lactide-co-caprolactone)
Starch	

Although natural polymers promise a high rate of clinical functionality, denaturation of natural polymers and the loss of their native structure during electrospinning have encouraged researchers to explore the usage of synthetic polymers as alternatives.[33] Synthetic polymers provide several advantages over natural polymers. They provide a wider range of mechanical properties and a desirable degradation rate. Several studies have been conducted for synthetic polymers such as PCL, PLA, PGA, PLGA [10, 24, 34-36]

1.7 Motivation for Choice of Materials

While a range of synthetic and natural polymers have been electrospun and the nanofibrous mats therefrom have been used for tissue engineering, this thesis focuses on how the scaffold surface properties affect cell growth and morphology. In order to do that, selection of materials is an important criteria. Four basic, commonly found electrospun materials, with different surface properties were chosen for this study to provide a better understanding on complex cell-substrate interactions.

1.7.1 Carbon. Research on carbon nanotubes and their applications in tissue engineering have been studied in depth. [37] However, the study of carbonaceous electrospun nanofibers is very limited in tissue engineering. Carbon nanofibers possess high Young's modulus and even electrical conductivity. [38-40] Carbon is common element in all living and non-living.

1.7.2 Cellulose. Cellulose is the most abundant polymer on Earth [41]. It is found on all plants and is essential for their survival. It is found in all plants and is the biomaterials that serves as the basis for the structural and mechanical support.[42, 43] Cellulose is also one of the most engineered biopolymers as the common component of wood, paper, cardboard and cellophane [41]. Cellulose is particularly interesting due to its availability and biodegradable nature.

Cellulose nanofiber applications in industry are mainly in filters, textiles and protective clothing [44, 45]. The hydrogen bonding in its structure lead to its insolubility in most of solvents [46]. In order to get cellulose nanofibers from electrospinning, cellulose acetate (CA) nanofibers may be first prepared and then treated with an alkaline solution for deacetylation. [47-49]

1.7.3 Cellulose acetate (CA). CA has been used for a wide range of uses from diaper material to membrane filters.[50] It has been shown that CA has antimicrobial properties. [51, 52] Applications of CA nanofibers include immobilization of enzymes and drugs, carrier for drug delivery applications, affinity membrane, hemodialysis and other biomedical applications.[53, 54] CA fibers have even shown photocatalytic properties. [55, 56]

1.7.4 Polyacrylonitrile (PAN). Polyacrylonitrile is a polymer with the formula $(C_3H_3N)_n$ Electrospun PAN fibers have good mechanical properties such as high thermal resistivity, elastic moduli and yield strengths [57, 58]. PAN is a common precursor for production of carbon. [45] It possesses unique thermal properties which can be utilized in optimizing textiles. [59]

The four types of electrospun nanofibrous mats, PAN, carbon, cellulose acetate and cellulose are easily available and more importantly, they stand for materials with different structure and surface property, which may have great influence on cells growth and morphology.

1.8 Epithelial Cells

Epithelium is the layer of cells that form the lining of organs and body cavities. They function as boundary elements serving important roles in development as well as in fully differentiated cells. They can act as sensory receptors. Epithelia are polarized cells containing a distinct apical and basal region. They rest on a basement membrane which is vascularized and connected to the tissue below. The intercellular space between these cells is limited. They form the tightest junctions in the world. Epithelial are polarized cells containing a distinct apical and

basal region. They rest on a basement membrane which is vascularized and connected to the tissue below.

Nanoscale topologies have seen to influence epithelial cell growth. [22] Studying the effects of epithelial cell growth and morphology based upon the substrate it is placed upon will allow researchers to better understand the workings of the cell. Cell-substrate interactions are not very well known nor understood with respect to epithelial cells. This preliminary study enables researchers to determine the starting point of scaffold design.

While very few studies regarding nanoscale features and their effects on epithelial cells have been studied in the past [22], even fewer have attempted to examine how they change based off of the base characteristics of the chosen scaffolds. In most tissue engineering studies, scaffolds are chosen based on availability and a few properties that have been understood about them. Rarely do they attempt to start from base characteristics and mold the cell growth based on scaffold properties. This preliminary study examines a few basic properties of electrospun scaffolds and how they could affect cell-substrate and cell-cell interactions.

1.8.1 Motivation for choosing Madin Darby Canine Kidney epithelial cells. Madin Darby Canine Kidney epithelial cells (MDCKs) were the cell line chosen for this study. They are mononucleate, non-transformed cells that form tight epithelial monolayers upon growth. These characteristics have been used to study transport phenomenon across barriers, tight junction formation and tubule formation [60-62]. MDCKs are an ideal representation of epithelial cells in the body due to these reasons.

CHAPTER 2

Literature Review

2.1 Applications of Scaffolds; Use In Vivo, To Generate Tissue Ex Vivo or In Vivo

2.1.1 Scaffold use in Tissue engineering. Although the emergence of tissue engineering has recently evolved to a level where it is now common medical practice, the precursors to this field have existed for some time - from early example such as the use of wooden dentures by George Washington to the modern day application nano calcium sulfate scaffolds for periodontal tissue engineering. [18] Charles Vacanti and Robert Langer brought to the forefront the field of tissue engineering with the creation of the Vacanti mouse [63]. Generation of tissue is this manner is an excellent example of how modern day scaffolds have been applied to field of tissue engineering.

2.1.2 Research on different scaffold materials. Over the evolutionary period of the tissue engineering field, the material from which scaffolds are comprised have changed drastically. These materials have improved from the classical use of wood to replace organs such as teeth and legs to a more biocompatible nature as found in wound healing and organ generation ex vivo. [1] Now with the applications of tissue engineering becoming more personalized, the materials used to generate scaffolds have a need to be further studied to allow for a greater degree of understanding and control in the therapeutic practice of patient care through tissue engineering. When choosing the material for the construction of the scaffolds, certain characteristics of the materials become of vital importance. The properties lend themselves to a more controllable and applicable scaffold able to achieve a more precise and desirable result. The key properties of the material examined when manufacturing the scaffolds are biocompatibility, immunocomplacency and surface topology [19]. These properties have been

studied by various researchers in an attempt to further lay the groundwork for the advancement of scaffold applications in tissue engineering.

In an effort to test some of the various desirable characteristics of scaffold material, a preliminary in vitro test has been carried out to ascertain the best scaffolds for use in vivo. Some of the factors that have been investigated pertaining to the cellular interactions with the scaffolding material are cell growth and proliferation, morphological changes, biocompatibility, etc. By varying just one of these factors, a marked change in cellular interaction may be observed. For example, in one study, the grooves on the surfaces of the scaffold fibers were changed to different parameters. Porcine epithelial cells were shown to migrate in the direction of the grooves while, while other cells types did not. [64] Alignment of BHKs and MDCKs was seen to increase with increased groove width and depth.

2.1.3 Nano topologies. Recent advances in the scientific field have been in the direction of nanotechnology. Nanotechnology has opened new avenues of research. Features are different at the nanoscale and affect cellular processes in manners more complex and diverse than from a macroscale perspective.

Nanostructures are also found in nature, from the adhesion pads of geckos to fullerenes in space. These structures are not without function, as is observed in the superhydrophobicity of lotus. The nano features may manifest from various geometrical conformations. Certain nanostructures such as the surfaces found on insect cuticles have shown to exhibit adverse effects on cells.[65] These geometrical nano structures may also be a part of the ECM.

2.1.4 Electrospun scaffolds. The most popular method of artificially manufacturing nano topologies is electrospinning. It offers several advantages, the most mentionable of these being the ability to choose the material, its cost effectiveness and ease of processing.

Since the conception of the electrospinning technique in 1897 by Rayleigh and the introduction of electrostatic charges to the process by Taylor in 1969, the scientific field has shown a remarkable interest in the technique. [66] Due to the increasing interest in nanotechnology, electrospinning has gained even more attention since the 1980's. Electrospun fibers have fascinated scientists as an alternative scaffolding material to those currently in existence. Much research has been conducted in this regard with applications ranging from filtration to clothing.

2.1.4.1 Materials used and different applications. The most common electrospun scaffolding material are synthetic and natural polymers such as PLA, PGA, PLLA, collagen, fibrinogen and gelatin.[67] Studies have encompassed the growth of cells on these scaffolds. The high surface area and porosity afforded by the electrospun nanofibers and the similarity of the base architecture to the native ECM, make electrospun fibers a good matrix for growing cells. [68] Stem cell research on electrospun scaffolds has shown that the nanotopologies offered by these electrospun scaffolds can influence differentiation into different cell types. [69]

2.1.4.2 Epithelial cells and Electrospun Nanofiber scaffolds. The interaction of epithelial cells with electrospun scaffolds that offer nano scale topologies is of importance to us. Topography on the nano scale greatly resemble in vivo morphologies. By changing this topography, we can influence and potentially control cell growth. Some research even indicated the change in motility due to nanotopologies.[70] Fiber topology of electrospun PLGA fibers has seen to significantly influence adult salivary mouse gland acinar epithelial cells in terms of adhesion and cellular morphology. [71] Some cells such as the NIH 3T3 cells grown on polyamide nanofibrous substrates were seen to induce contact guidance and influence multicellular spheroid formation. [36] Electrospun PLAGA surfaces showed increased

epithelialization [72, 73]. Change of topography may also be in the form of mechanical stress from the fibers. These stresses, such as fiber alignment, play an important role in influencing cell growth. For example, a recent study shows that human ligament fibroblasts show spindle shape along aligned polyurethane fibers and secreted more ECM than when attached to random fiber configurations. [74]

2.2 Materials

Electrospun carbon nanofibers have shown varied data in relation to the growth and proliferation of cells. Several studies conducted osteoblasts, showed increased adhesion and good cytocompatibility. [38, 40, 75] However, with other cell lines such as fibroblasts and astrocytes, a decreased function and even cellular damage was seen [38, 76].

Human fibroblasts and keratinocytes have shown a short term culture of cells [77]. Cellulose nanofiber matrices, while biocompatible [77], have shown good cellular penetration with respect to MC 3T3 osteoprogenitors [78], clustering of osteoblasts with cytoskeletal growth and stretching [79].

PAN surfaces, although non-toxic in nature have been used as substrates to grow 3T3 cells was shown to have a fewer number of cells and a smaller size. [80] The use of electrospun PAN surfaces on MDCKs on the other hand, supported attachment and proliferation. [77, 81] While the nanofibrous nature of ES PAN Surfaces has seen to be advantageous, there is not enough conclusive evidence to show

2.3 Importance of this Study

Although studies have been performed to gauge the functionality, morphology and viability using various substrate materials, certain considerations must be taken into account. When examining the results of the aforementioned studies, it is seen that the fundamental

properties of the substrate materials deserve further examination. This study explores some of the basic properties belonging to four commonly used materials chosen in the generation of TE scaffolds: PAN, Carbon, CA, and Cellulose. By utilizing the same cell line on the four various materials, a comparative study now allows for exploration into inherent interaction between these substrates and cellular functionality. The lack of coherent data points out three major concerns deserving of further scientific study: the various considerations for the choice of material substrates, variability of cell type choice resulting in ambiguity in substrate-cell interactions, lack of concern for the manufacturing of scaffolds leaves question to the bottom-up design of scaffolds using various materials.

Different electrospun fibers induce different changes in varied cell types as can be seen from the examples mentioned above. These changes could be due to a number of reasons. This research focusses on documenting the changes observed in epithelial cell line MDCK based upon three fundamental characteristics: choice of material, range of nanofiber sizes and the wetting properties of the fibers.

The purpose of this study is to draw attention to the questions that remain after analysis of the aforementioned studies. The interactions between a singular cell line and a variety of nanofibrous substrates is meaningful and fundamental for future tissue engineering.

CHAPTER 3

Methodology

3.1 Electrospinning

The electrospun materials for this study were obtained from the Materials Manufacturing Lab. The original solution parameters are as follows:

3.1.1 Polyacrylonitrile (PAN) nanofibers. PAN solution prepared by mixing 10 wt. % PAN in DMF at 70 °C with constant stirring. Electrospinning of solution at voltage of 15 KV and flow rate of 1.2 ml/hr.

3.1.2 Carbon nanofibers. Carbon nanofibers were prepared from PAN nanofibers through stabilization to degrade the polymer and carbonization at high temperatures in the presence of nitrogen gas.

3.1.3 Cellulose Acetate nanofibers (CA). The solution containing 40 g Tetrahydrofuran, 40 g Dimethyl Sulfoxide, 0.1 g Sodium Citrate, and 20 g Cellulose Acetate was spun at a rate of 1.2 ml/hr. and 22 KV voltages.

3.1.4 Cellulose nanofibers. Cellulose nanofibers were prepared through the deacetylation of electrospun CA done by overnight reaction in 0.05 M sodium hydroxide (NaOH).

3.2 Cell Culture

3.2.1 Cell culture maintenance. Madin Darby Canine Kidney epithelial cells were obtained from the Adamson Laboratory at UNCG. They were maintained in tissue culture flasks with nutrient media containing high Glucose DMEM with 10% FBS and 1% pen-strep antibiotic cocktail in 75 cm^2 cell culture flasks at 37 ° C. The flasks are passaged regularly upon reaching

90% confluency for maintenance. Media was changed every two days to provide a healthy environment.

3.2.2 Cell seeding onto electrospun surfaces. The electrospun surfaces were cut into 1cm x 1cm squares. After UV sterilization for 2 hours and subsequent sterilization with 70% alcohol, the surfaces were deemed ready for cell culture. The electrospun surfaces were placed at the bottom of 24-well plates for tissue culture. MDCKs were seeded onto each of the four electrospun surfaces and a glass coverslip which was used as a control at a seeding density of 0.11 x 106 cells for an area of 1.9 cm2.

3.2.2.1 Cell fixation and staining protocol for immunofluorescence. MDCK cells were grown on the electrospun surfaces and glass coverslip placed in the 24-well tissue culture plate over 4 days at 37˚C. Regular growth medium**,** high Glucose DMEM plus 10% fetal bovine serum and 1% Pen-Strep Antibiotic cocktail, was provided to ensure optimal growth. This was the time required for the control to achieve confluency. The cells were fixed after incubating with freshly prepared 4% paraformaldehyde solution for 20 minutes. PBT blocking solution (0.1 % Triton X-100 in 10 ml of 1x PBS and 1% BSA) and incubation for an hour allowed the cells to be permeabilized. The surfaces was further incubated with primary antibody, anti-cadherin at 1 : 1000 dilution in 1x PBS plus 1 mg/ml BSA for 30 minutes followed by incubation at room temperature, in the dark. The secondary antibody (Cy3-AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG, Product code JAC-115166062) at 1:2000 dilution in PBS plus 1 mg/ml BSA was added after this step for an hour-long incubation. The Alexa Fluor® 488 dye was added at this point and left for another hour. The Hoechst nuclear dye was allowed to sit for 5 minutes. Every step was followed by a thorough rinse using 1x PBS. The surfaces were mounted on a glass slide

with the help of a mounting medium (Aqua Poly/Mount, Cat. # 18606) and stored in the dark until ready to be imaged under the microscope.

3.3 Characterization Techniques

The electrospun fibers obtained were characterized using two techniques: SEM and Contact Angle measurements. These analyses were performed to fully understand the properties of the fibers in question.

3.3.1 SEM analysis. Scanning electron microscope is an instrument used in image characterization of materials at the nanoscale. Electrons are emitted at a high speed upon a singular spot on the sample which knocks loose a number of electrons from the spot. These electrons are collected and further interpreted by the microscope to provide an image of the topography of the sample placed under the objective. This requires the sample to be fairly conductive and have the least amount of charge possible to ensure a reliable output. In order to view the Cellulose, Cellulose Acetate and PAN electrospun membranes under the Zeiss Auriga FIB/SEM, the samples were sputter coated, using a Leica EM ACE200 with real-time thickness monitoring using a QCM with a conductive material. These samples were sputter coated with gold at a thickness of 5 nm. All the samples were then viewed under the SEM under vacuum for imaging and analysis. Images were acquired at similar magnifications (1x and 15x).

3.3.2 Contact angle. The instrument known as a goniometer measures the contact angle of a water droplet on a sample surface. This yields information regarding the wettability of the surface. Water-in-air (WIA) contact angle can be directly related to how tissues behave on a substrate. It has been shown to be linked with attachment, spreading and growth of cells on the substrate. [82] . The contact angle of distilled water on each of the electrospun surfaces was measured according to the following procedure.

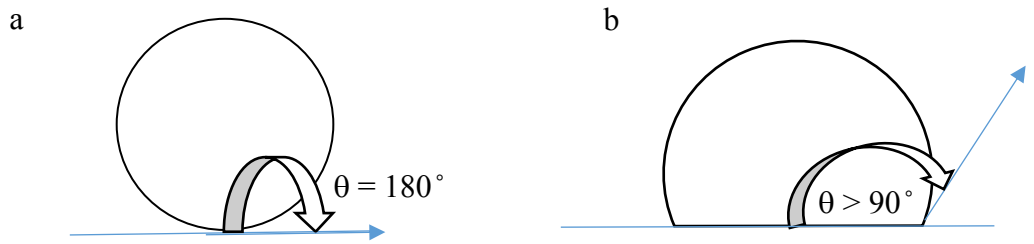


Figure 3-1. Depiction of contact angle for a) hydrophobic substance (θ > 90˚) b) hydrophilic substance (θ < 90˚)

The electrospun samples were mounted on glass slides with double sided sticky tape. The samples were placed in between the light and camera of the setup, a Rame´-Hart 260-F4 contact angle goniometer with DROPIMAGE advanced software. 5 µl of water was then dropped onto the surface using a pipette and imaged. Contact angle was measured after 15 seconds. This was the time required for the water droplet to stop spreading. The angles were measured for both the advancing and receding contact angles and averaged. The average was sampled for 10 different droplets of water onto the surfaces. The contact angle is measured from a baseline to the tangent of the water droplet. If the angle is less than 90˚, the sample is hydrophilic in nature while it is said to be hydrophobic in nature if the angle is greater than 90˚. A perfectly hydrophobic material has a contact angle of 180˚.

3.4 Confocal Assays.

The main method of analyses of cell-substrate and cell-cell interaction in this study was the use of confocal microscopy. This is a versatile microscope that allows the user several unique functions. It measures fluorescence of a sample at specified wavelengths and converts these into discernable images on the screen for further analyses. A Zeiss Axio Plan spinning

disc confocal microscope with FITC excitation filter at 495 nm and emission filter at 535 +/- 20 nm for Alexa Fluor® 488, and an excitation filter at 355 +/- 20 nm and emission filter at 440 +/- 20 nm for DAPI was used.

3.4.1 Cell counting. MDCK cells were stained for their nuclei using the Hoechst dye as mentioned previously. These cells being mononucleate provide the advantage of being able to discern the number of cells per unit area visually by counting the number of nuclei depicted on the confocal imaging software. Cells were labelled with Hoechst dye which fluoresces at 360 nm wavelength to label the nucleus.

3.4.2 Viability assay. The live dead cells assay was performed using the Acridine Orange Propidium Iodide method. This is a sensitive colorimetric assay that allows the determination of the number of viable cells. Acridine orange is a nuclear stain that emits a green fluorescence (525 nm) upon binding with double-stranded DNA and red (>630 nm) upon binding with RNA. Propidium iodide intercalates between the DNA bases in fixed cells to emit a red fluorescence. Cell monolayers growing on our prepared surfaces in 24-well plates were washed thrice with PBS and stained with 0.1 % acridine orange made in Ringer's solution and propidium iodide (0.1 mg/ml). They were incubated for 30 min at 37˚C. The liquid was then removed and the coverslip was placed on a larger coverslip with mounting media. They were viewed immediately under the microscope.

3.4.3 Morphology. By viewing the actin cytoskeleton under the appropriate channel under the microscope at 63x oil objective, the morphology of the cells can be ascertained. Actin filaments are stained for using phalloidin 488 stain which emits fluorescence at 495 nm wavelength. F-actin protein is found on the surface of all eukaryotic cells. It exhibits polarity, thereby aligning itself in a particular fashion. It is involved in processes like cellular

morphogenesis and cell migration. The overall morphology of the MDCKs was determined based on cell clustering and individual cell morphology.

3.4.4 Cell Area/ Spreading. The F-actin stain serves a dual purpose in enabling us to determine the area occupied by the cell. The confocal microscopy has a function which allows the user to determine cellular area by tracing the outline of the cell and running the units based off of the scale. Cellular area of 15 different cells present on each electrospun surface was evaluated using the Z-stack images.

3.4.5 Cadherin Analysis. The cadherin protein is an adherens junction protein found in the cell-cell junctions. During fixation and staining, cells were stained specifically using anti-cadherin antibody. The amount of cadherin present in these junction was determined using a densitometry assay. Yet another function of the confocal microscope is to allow users to determine the density of pixels per unit area. By outlining the areas of interest along the cell junctions, the pixel count for that particular area was obtained and compared.

CHAPTER 4

Results

This chapter deals with the results obtained from the various experiments that have been carried out for this study.

4.1 Characterization:

4.1.1 Characterization: SEM analysis

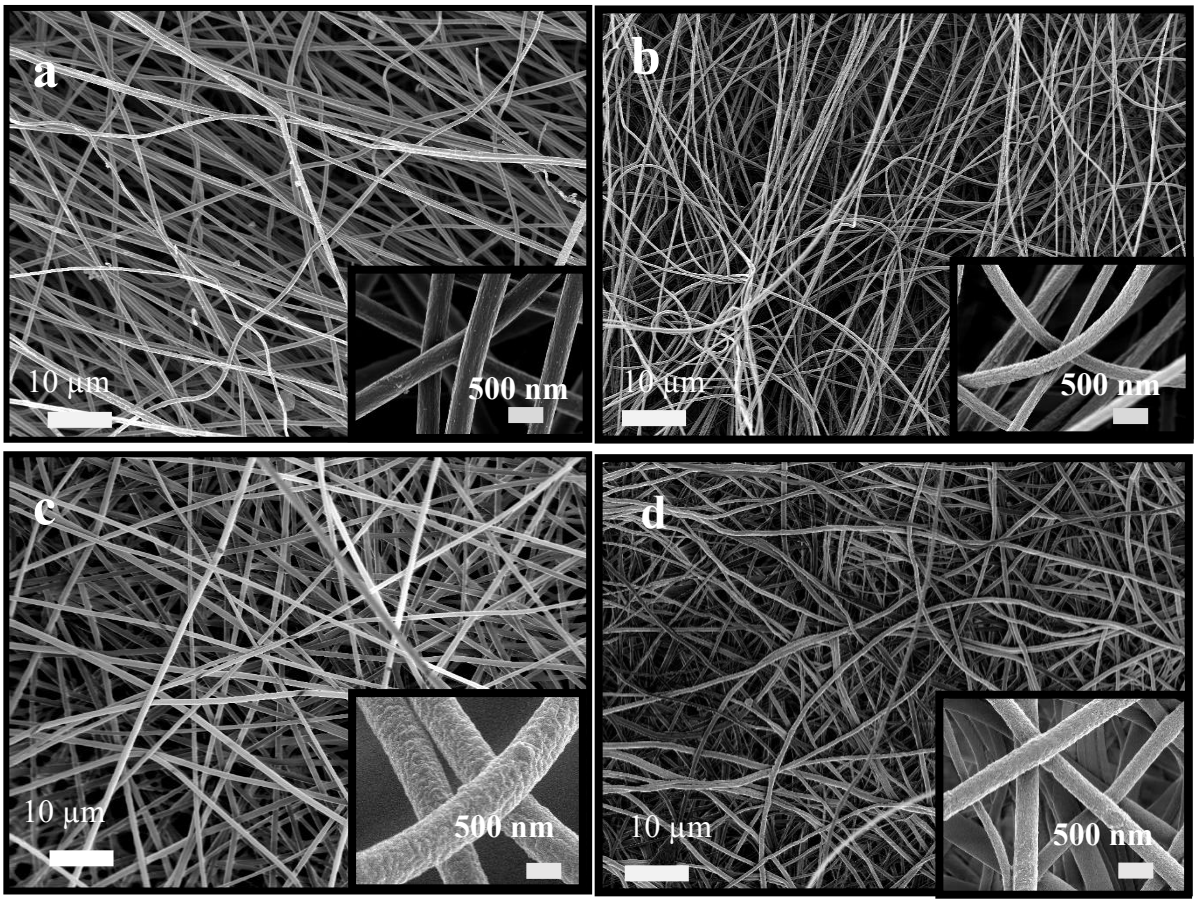


Figure 4-1. SEM micrographs of electrospun a) Carbon b) PAN c) Cellulose d) CA fibers at 1x magnification. (insets: at 15x magnification)

SEM analysis of the electrospun fibers has been summarized in Table 4.1 below.

Table 4-1

Summary of width analysis of the electrospun fibers using the SEM

	CARBON	CELLULOSE	CELLULOSE ACETATE	PAN
Mean	750.057	1017.9	603.196	452.12
Standard Deviation	74.98	190.31	221.16	56.46

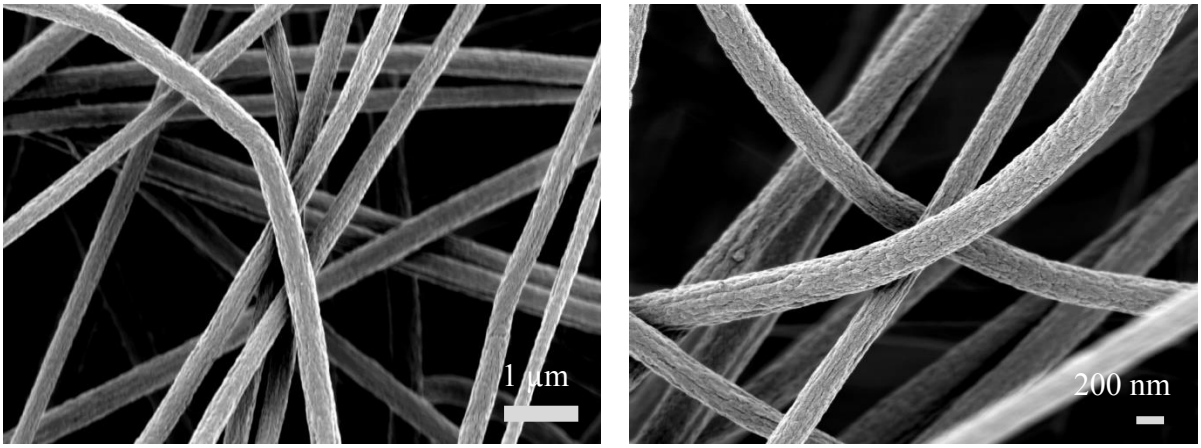


Figure 4-2. SEM micrographs of PAN fiber at a) 10x and b) 15x magnification

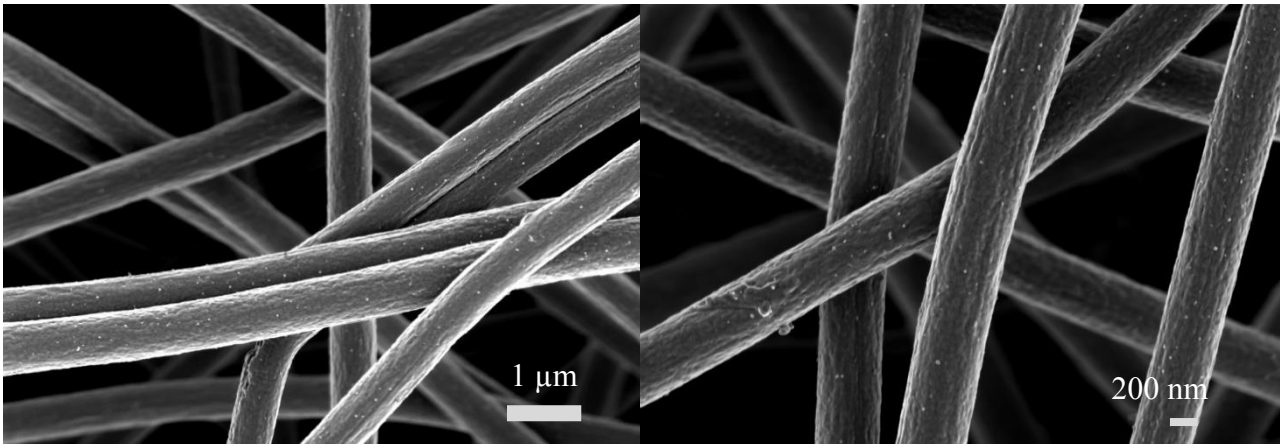


Figure 4-3. SEM micrographs of Carbon fiber at a) 10x and b) 15x magnification

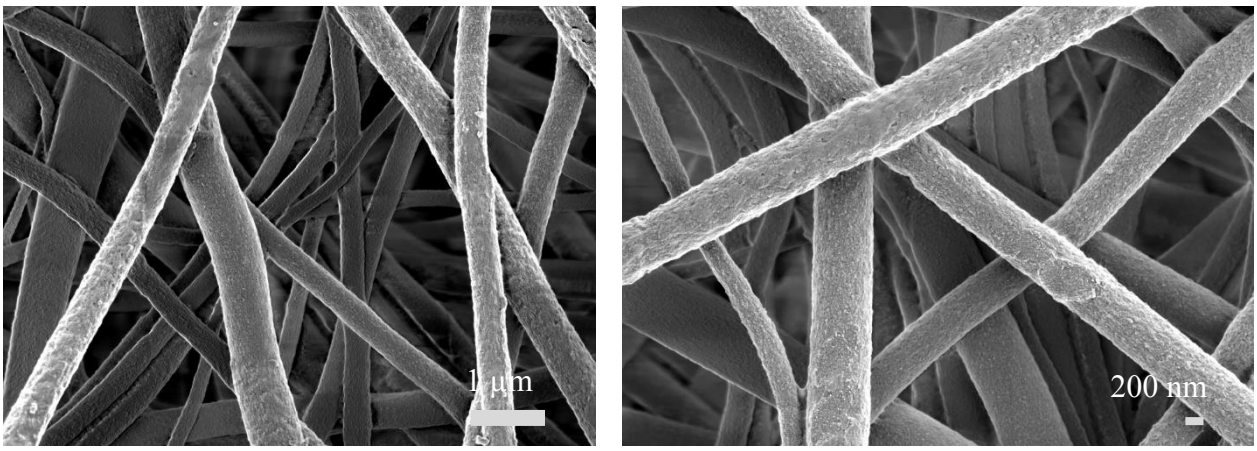


Figure 4-4. SEM micrographs of CA fiber at a) 10x and b) 15x magnification

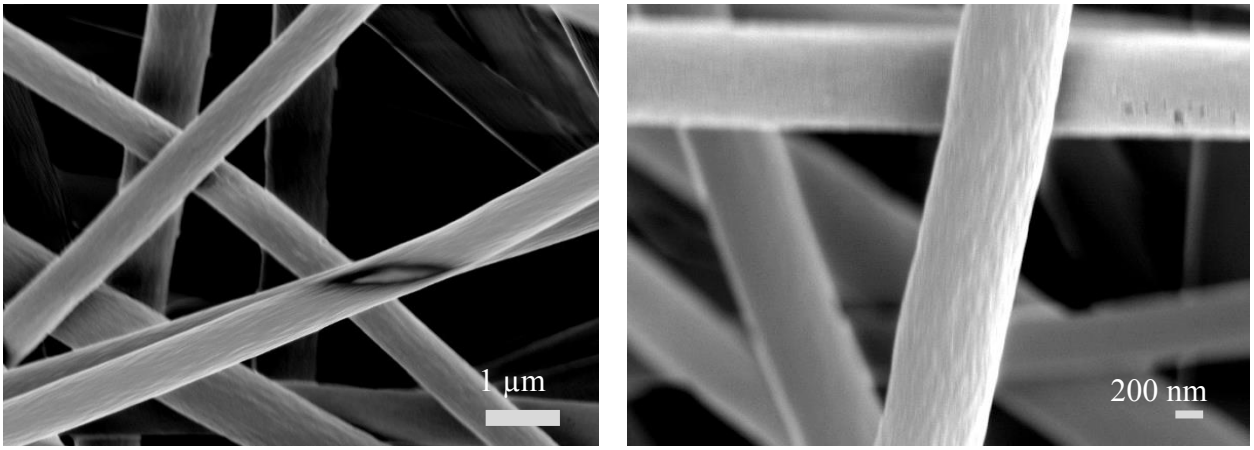


Figure 4-5. SEM micrographs of Cellulose fiber at a) 10x and b) 15x magnification

It can be seen that Cellulose fibers were the largest amongst the electrospun fibers at an average width of 1017.9 ± 190.31 nm. The smallest electrospun nanofiber was Carbon with an average width of 750.057 ± 74.98 nm. Polyacrylonitrile (PAN) nanofibers were found to be in the range of 452.12 ± 56.46 nm. Cellulose Acetate (CA) nanofibers were found to have an average width of 603.196 ± 221.16. CA was by far the most varying fiber in terms of width. There was no uniformity in size, unlike the PAN and Carbon nanofibers which were seen to have a comparatively uniform distribution of width. The SEM micrographs show no apparent orientation of the fibers. There was no sign of uniform distribution of the electrospun nanofibers. The greater width of the Cellulose nanofibers provides a greater surface area for cellular

interaction. From the data collected, it is seen that Carbon nanofibers have the smallest width. This can have potential toxic effects on cells. The size is small enough to pierce certain vital organelles. The fibers show a wide range of sizes and morphological characteristics.

Upon examining the SEM micrographs, different surface topologies were noted on each of the different types of electrospun nanofibers. Grooves were seen to be present on the surface of PAN, CA and Carbon nanofibers. Cellulose nanofibers were seen to be distinctly smoother and less grooved. Carbon nanofibers showed a number of surface particles. Since it is not possible to discern the beginning of the fibers from the end, length was not determined.

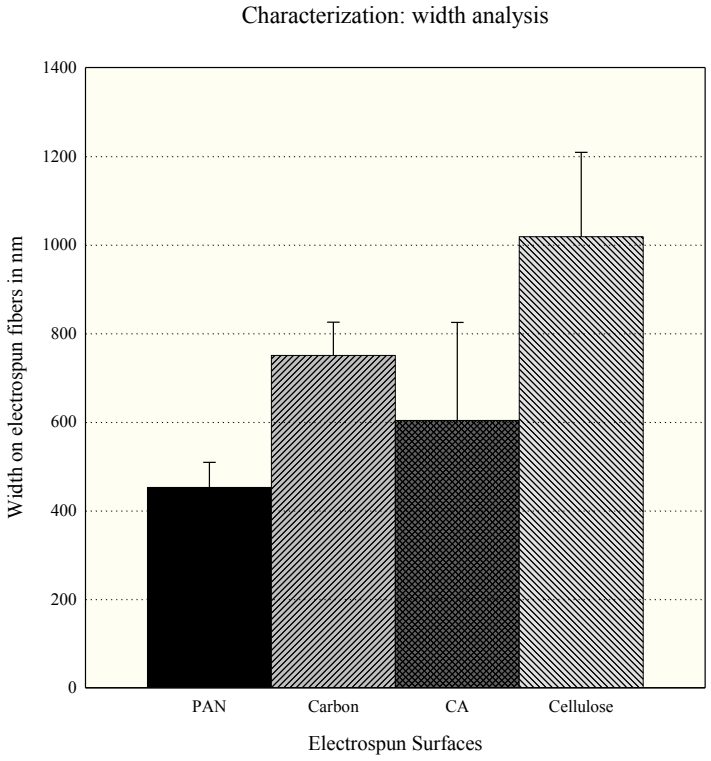


Figure 4-6. Graph indicating the average width of the electrospun fiber surfaces

4.1.2 Characterization: Contact Angle Measurements. Contact angle measurements were made using the imaging software attached to the goniometer. These measurements enable us to understand the wettability of a surface. Larger contact angles correspond to a more

hydrophobic surface while smaller contact angles correspond to hydrophilic surfaces. Optimal contact angle measurements for cell adhesion is between 50 and 100 degrees. Images of contact angle for water on each of the electrospun surfaces and the control is shown in Fig (). The image has been arranged in order of decreasing contact angle per surface.

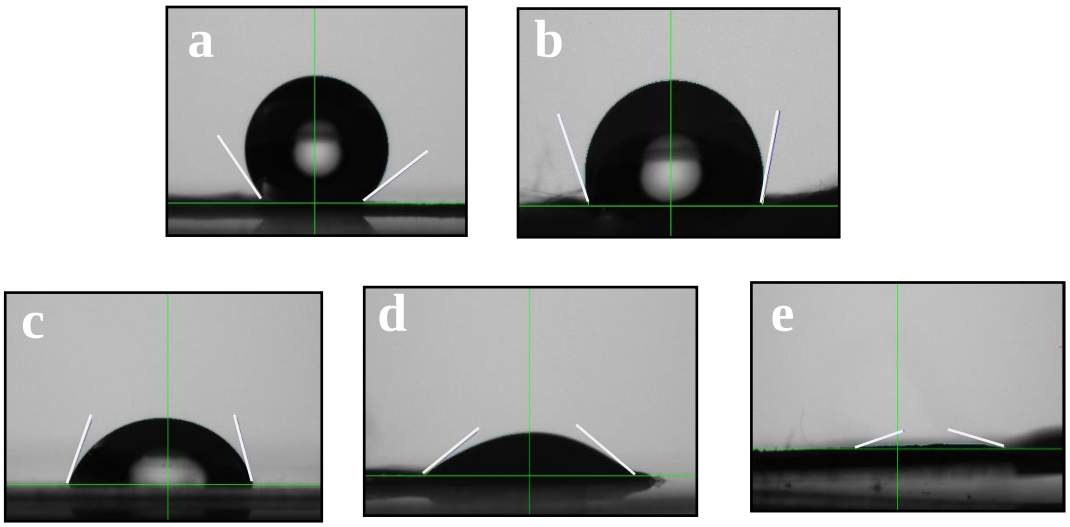


Figure 4-7. Contact angles of various electrospun surfaces a) Carbon b) Cellulose acetate c) Control glass cover slip d) Polyacrylonitrile (PAN) e) Cellulose

Table 4.2 represents the summary of the water-in-air contact angles. A total of ten measurements was taken and averaged at different points on the surfaces. The highest values of contact angle were recorded for electrospun Carbon, while the least was for electrospun cellulose. It should be noted that contact angle values for electrospun surfaces are different from the natural material values. The contact angle values of all the surfaces are over a broad range of values. This gives us a good spectrum for understanding cell attachment and interaction based on wettability of the surface.

Table 4-2

Summary of Contact angle on electrospun surfaces

Sample	**water-in-air contact angle**
Glass coverslip used as control	71.18 ± 3.52
PAN	33.58 ± 0.07
Carbon	143.26 ± 0.07
Cellulose Acetate	107.75 ± 7.83
Cellulose	13.445 ± 0.81

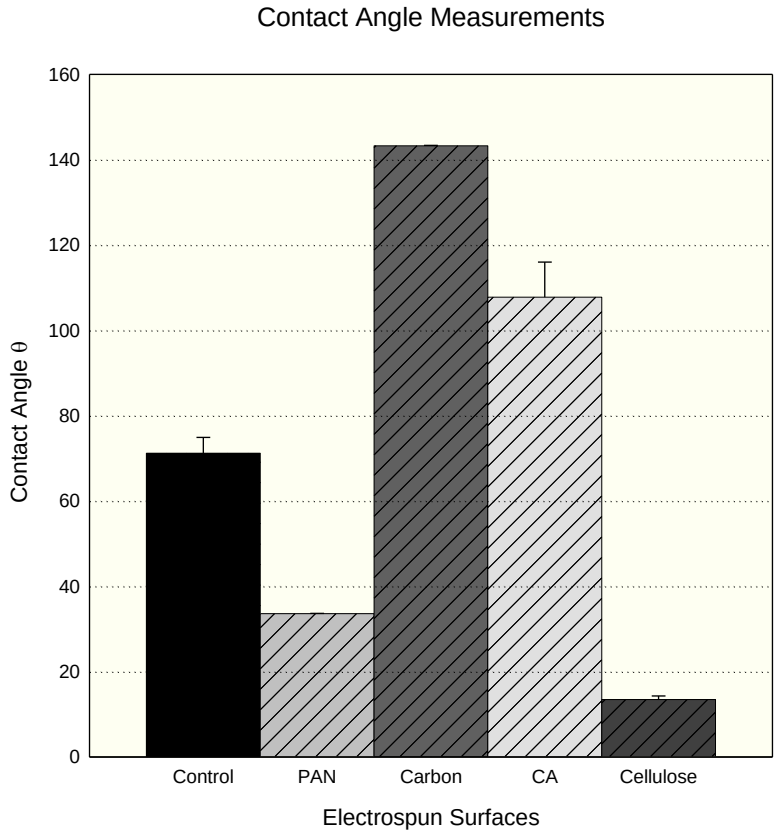


Figure 4-8. Graph of Average contact angles on the electrospun surfaces

4.2 Assays

4.2.1 Cell counting assay

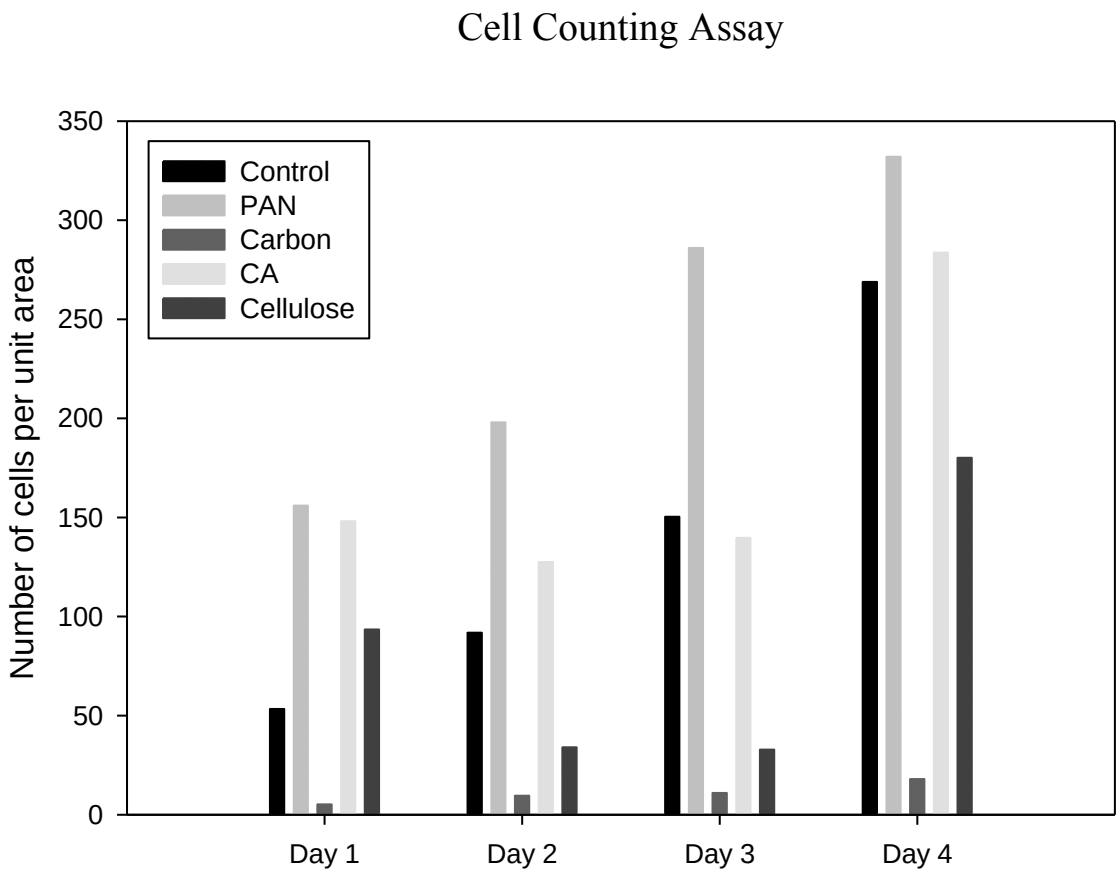


Figure 4-9. Graph showing the number of cells per unit area from day 1- day 4 of cell growth.

Images taken on a fluorescent scope of Hoechst stained nuclei of the MDCKs were visually counted at 9 random locations on each of the electrospun surfaces and the glass cover slip control. The number of nuclei correspond to the number of cells on the surface of each surface. The cell count on the control surfaces was seen to double with the passage of each day. The electrospun PAN surface consistently showed the greatest number of cells. Electrospun cellulose surfaces showed a small dip in the number of MDCKs before increasing between the third and fourth day of growth. Similarly, the electrospun cellulose scaffold shoed a dip in the number of cells from day 1 to day 3 before showing an increase by the fourth day. According to

the data presented in this assay, it can be surmised that the PAN surface promotes the best cell growth in terms of numbers.

4.2.2 Viability assay. The acridine orange propidium iodide method of analysis of viability of cells on each electrospun membrane yielded the following data. Cell viability was determined in percentage form using the following formulae:

Equation 1

$$\%\ Live\ cells = \frac{live\ cells}{live\ cells + dead\ cells} * 100$$

Equation 2

$$\%\ Dead\ cells = \frac{dead\ cells}{live\ cells + dead\ cells} * 100$$

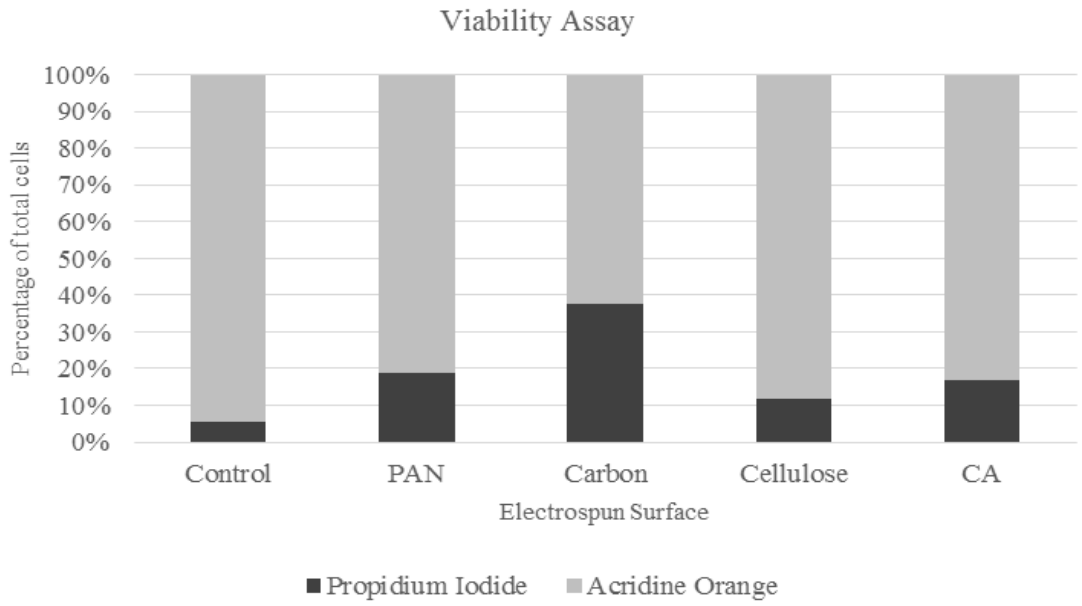


Figure 4-10. Graph showing the percentage of live and dead MDCKs

Table 4-3

Percentage values of live and dead cells obtained through Acridine Orange Propidium Iodide staining

	Propidium Iodide	**Acridine Orange**
Control	5.67	94.32
PAN	18.81	81.18
Carbon	37.72	62.28
Cellulose	11.58	88.41
CA	17.03	82.96

Fluorescent scope images from confocal microscopy were analyzed to count the number of live and dead cells. This data was further converted to percentage of viable cells. The data from the cell counting assay was corroborated and supplemented by the results of the viability assay. A high percentage of MDCKs was seen to be non-viable on electrospun carbon surfaces. The control surface showed the least death percentage. This follows with our expectations since the glass cover slip is nearly smooth and does not have many features to hinder cell growth. Electrospun PAN and Cellulose surfaces promoted 81% and 88 % growth.

4.2.3 Analysis of morphology of the cells. Morphology of MDCKs was examined by analyzing the F-Actin cytoskeleton.

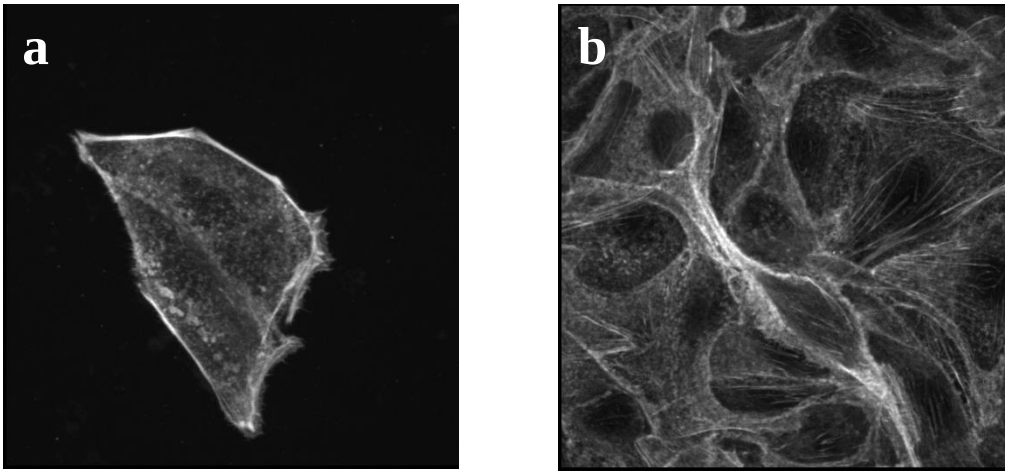


Figure 4-11. Confocal images on MDCKs grown on control surfaces a) day 1 b) day 4

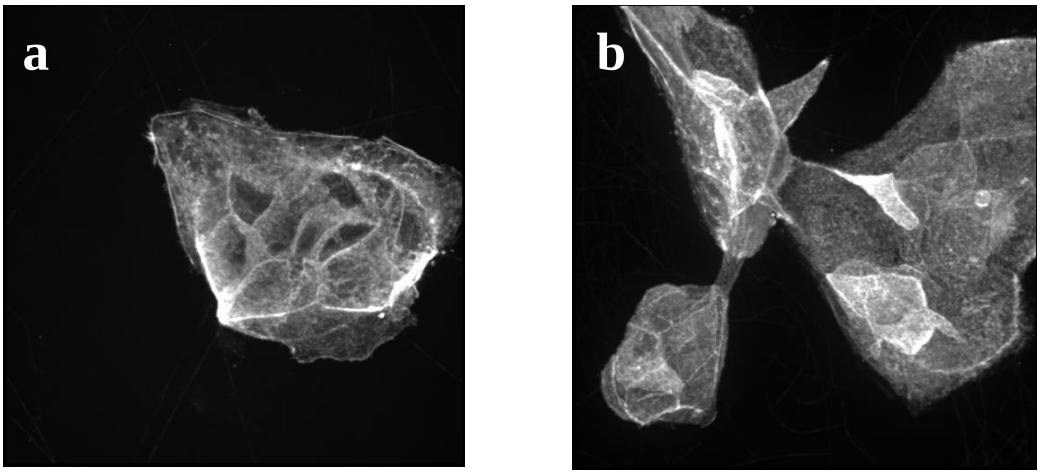


Figure 4-12. Morphology of MDCKs grown on electrospun PAN surfaces on a) Day 1 and b) Day 4

The images obtained from confocal microscopy indicate that the MDCKs have a 2-dimensional, flattened morphology typical of the MDCKs. In fig b, actin stress filaments can be seen clearly. MDCKs were seen to be flat and spread. By analyzing z-stacks, it was seen that the stress filaments were on the bottom section attached to the basement membrane.

The morphology of MDCK cultured on PAN surfaces assumed a distinct flat, elongated form. A change from flattened to slightly rounded and elongation happened between day 1 to day 4. Cells were found to be interlaced and stacked with the electrospun nanofibers in great numbers.

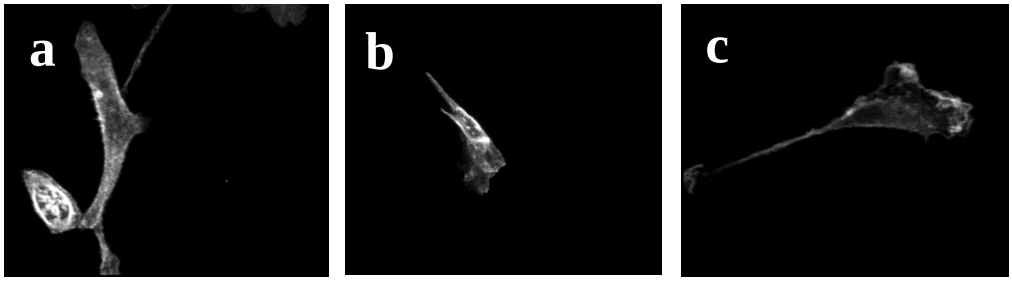
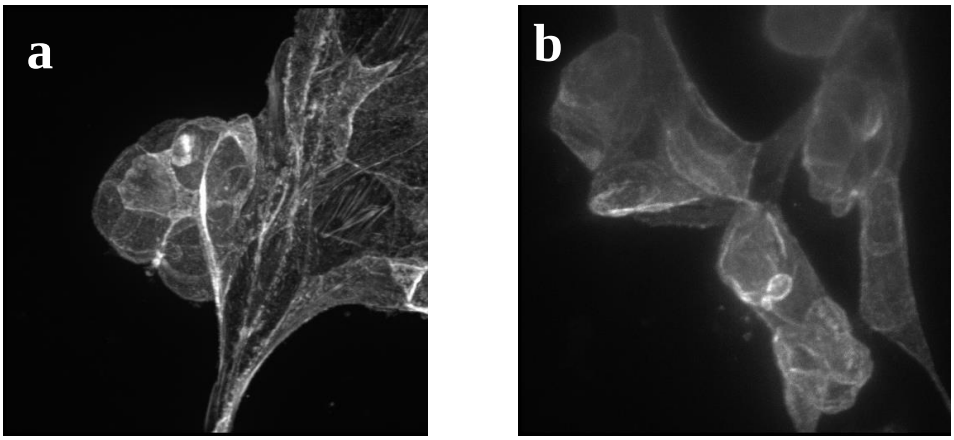


Figure 4-13. Morphology of MDCKs cultured on electrospun carbon surface representative of a) Day 1 and b) Day 4 c) petal-like morphology of cells seen on the periphery of the electrospun carbon mats

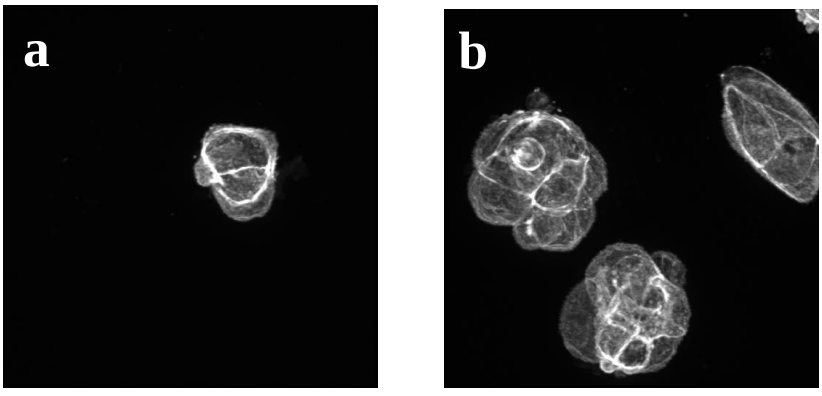
A tapering morphology of cells was observed on the cells cultured on electrospun carbon surfaces. A change in morphology with rupturing of actin cytoskeleton was observed. The MDCKs did not have a controlled growth. The cells found on the perimeter of the electrospun carbon surface was seen to vary in shape with grooves and edges. A distinct petal shape was seen in these cells. Most MDCKs grown on electrospun carbon surface exhibited shrinkage followed



by cellular rupturing.

Figure 4-14. Morphology of MDCKs grown on CA surface on a) Day 1 and b) Day 4

Electrospun Cellulose acetate nanofibers encouraged rounding of the cytoskeleton of the MDCKs. An increase in balling of the cells was observed between day 1 and day 4. However, it



was not a complete balling, with some of the original flat cells remaining.

Figure 4-15. Morphology of MDCKs cultured on electrospun cellulose nanofiber surface on a) Day 1 and b) Day 4

Electrospun cellulose surfaces heavily encouraged the formation of balls. MDCKs tended to form close interactions with neighboring MDCK cells. Individual cell morphology was seen to be circular. MDCKs were found to form clusters of cells. These clusters also had a spherical morphology.

4.2.4 Cell area and spreading

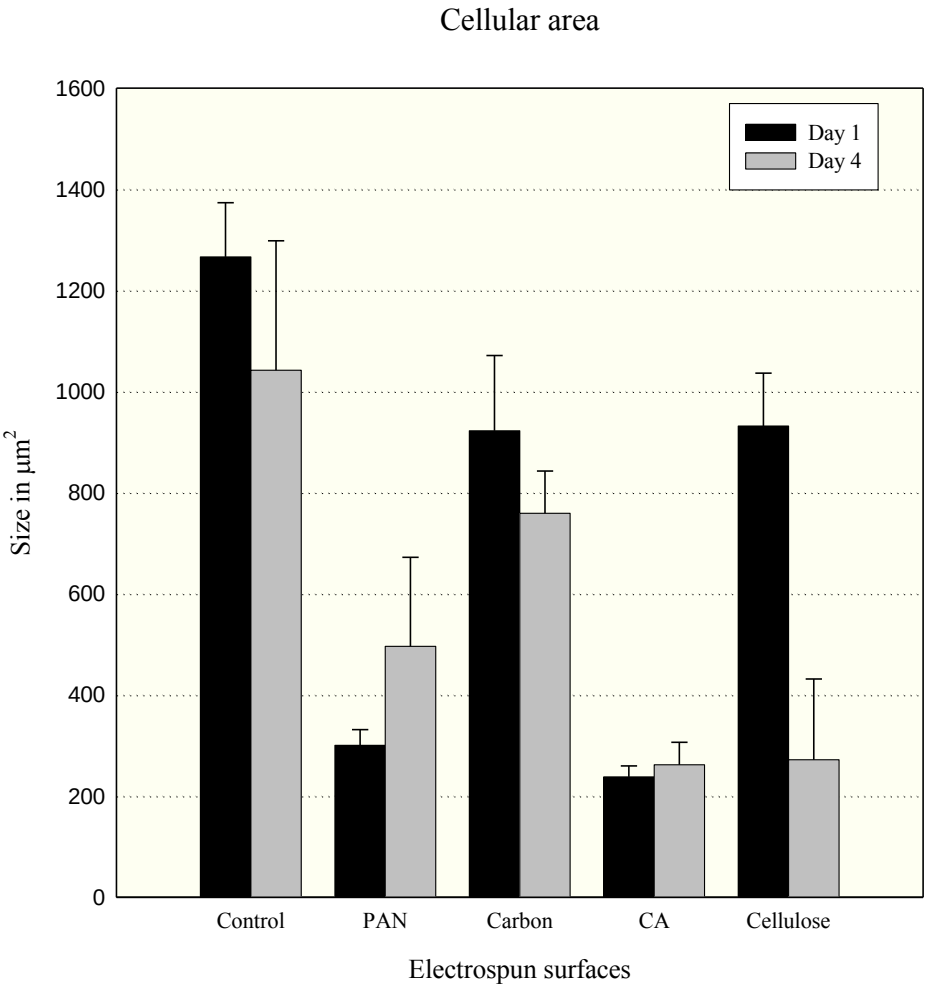


Figure 4-16. Graph indicating change in area of MDCKs from Day 1 to Day 4 of cell culture on the electrospun surfaces.

The changes in morphology were quantified by analyzing the 2D surface area taken up by the cells on each surface. The results of this analysis are depicted in the graph above.

Control surfaces had a larger cellular area as expected. This can be attributed to the 2 dimensionality of the glass control. A decrease in the area of MDCKs between day 1 and day 4 is due to cells contracting to accommodate new cells. PAN and CA have shown an opposite effect on MDCKs. Electrospun carbon showed a decrease in MDCK area. This is probably due to the

rupturing of cells on this surface. Electrospun cellulose fiber surface shows a great decrease in area and spreading due to the rounding up of cells and the formation of clusters in a ball shape. PAN surfaces were found to cause an alignment of cells in the direction of majoority of the fibers. This phenomenon is called contact guidance.

4.2.5 Adherens junction protein quantification

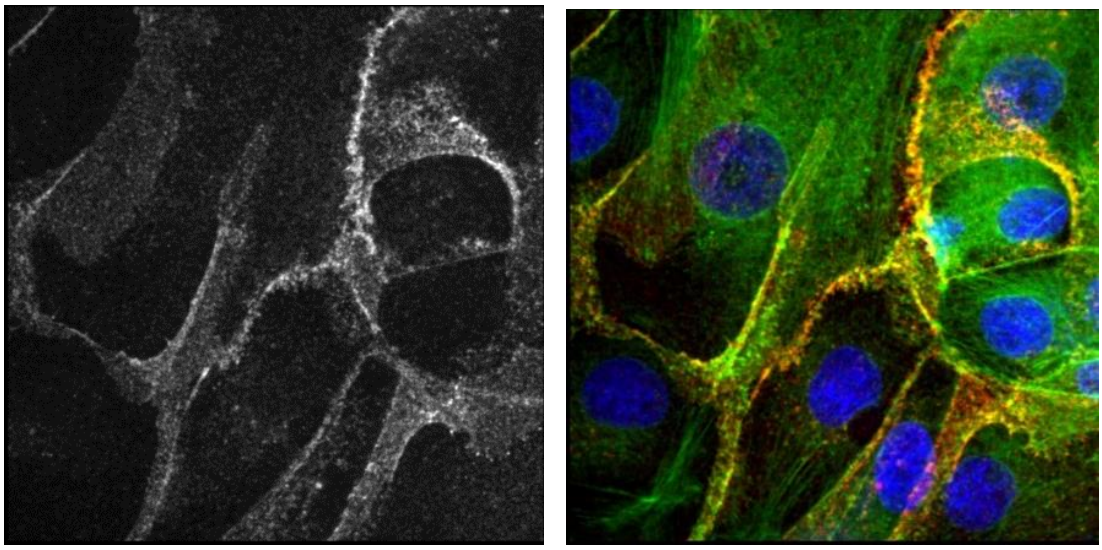


Figure 4-17. Adherens junction protein, cadherin, as seen in MDCKs cultured upon control surfaces. Left: through red channel. Right: hrough all channels

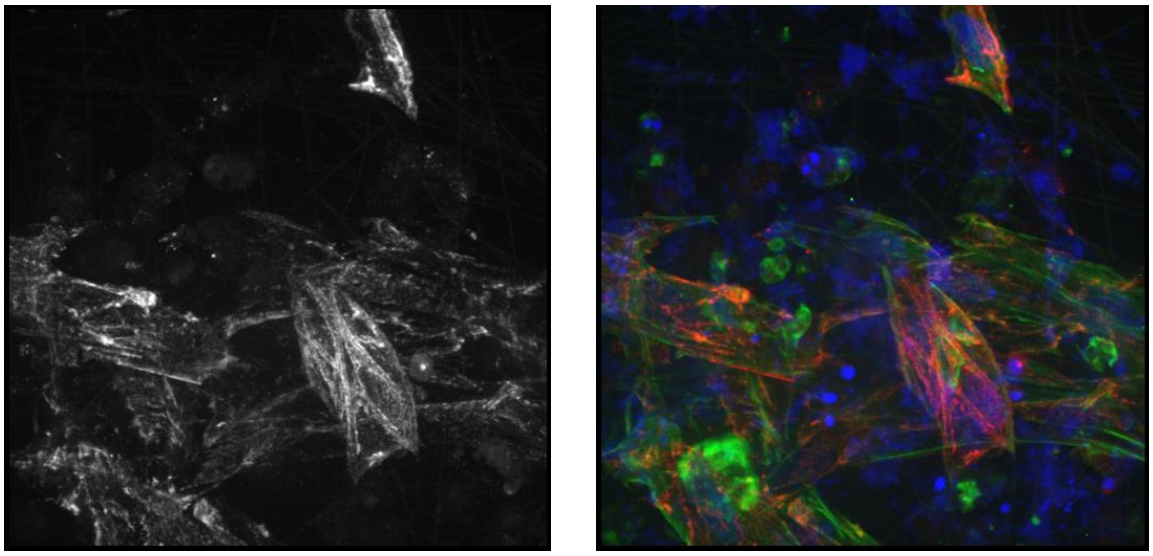


Figure 4-18. Adherens junction protein, cadherin, as seen in MDCKs cultured upon electrospun PAN surfaces. Left: through red channel. Right: hrough all channels

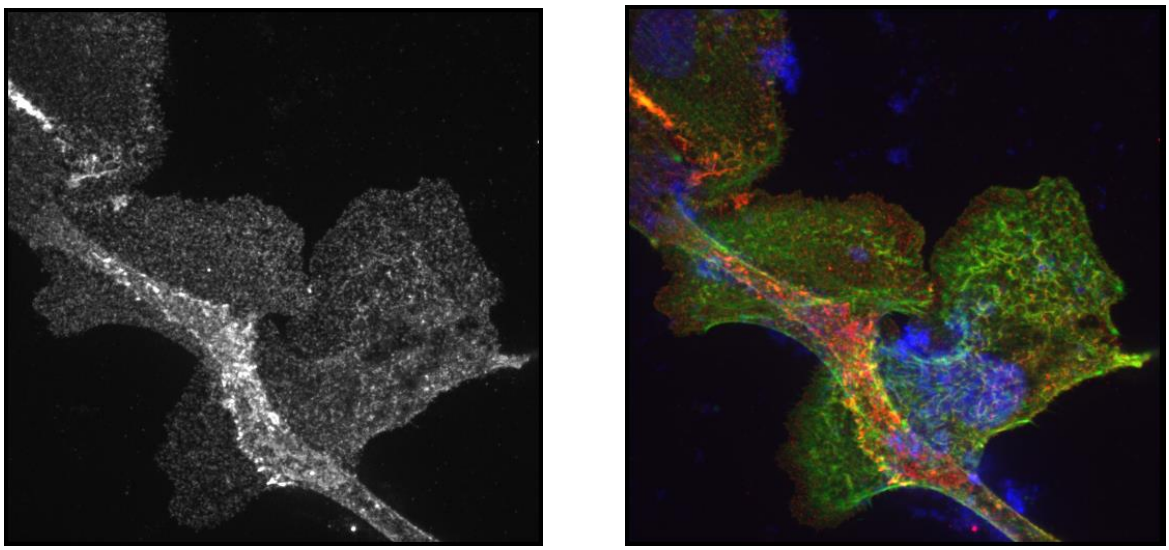


Figure 4-19. Adherens junction protein, cadherin, as seen in MDCKs cultured upon electrospun Carbon surfaces. Left: through red channel. Right: hrough all channels

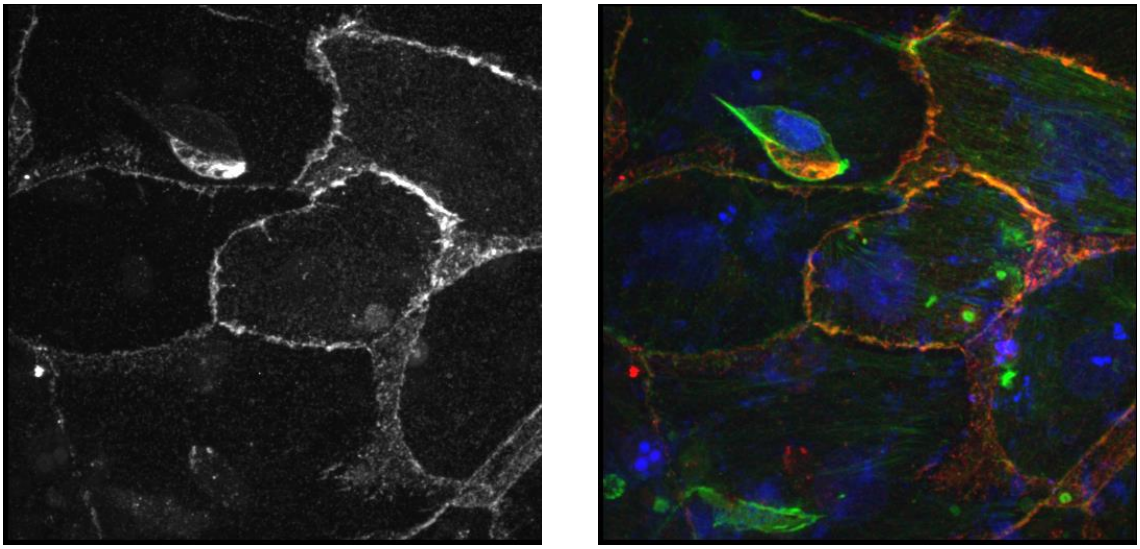


Figure 4-20. Adherens junction protein, cadherin, as seen in MDCKs cultured upon electrospun CA surfaces. Left: through red channel. Right: hrough all channels

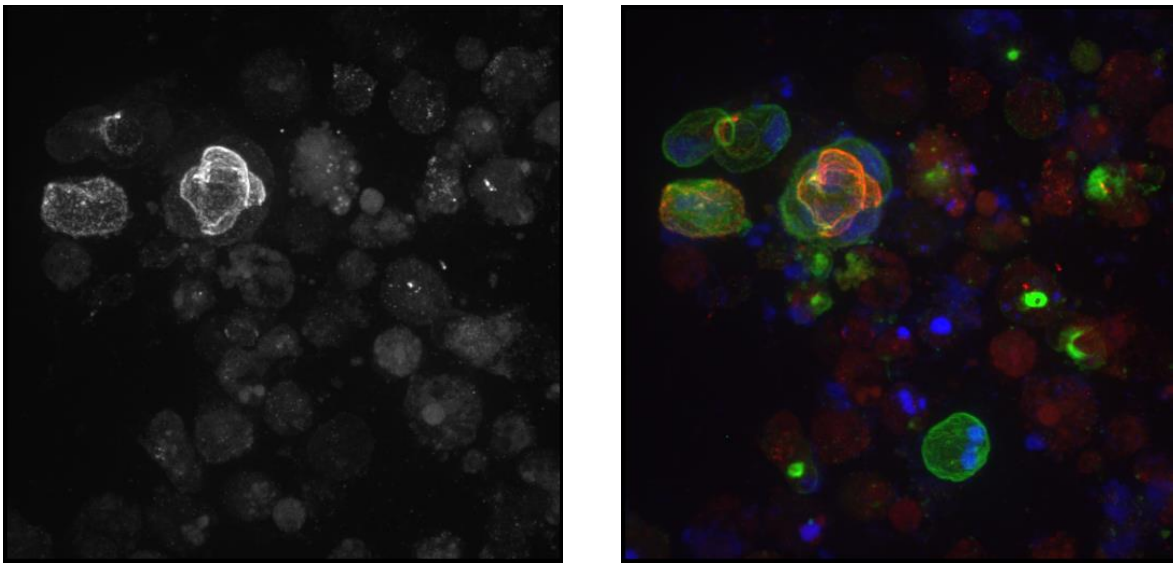


Figure 4-21. Adherens junction protein, cadherin, as seen in MDCKs cultured upon electrospun Cellulose surfaces. Left: through red channel. Right: hrough all channels

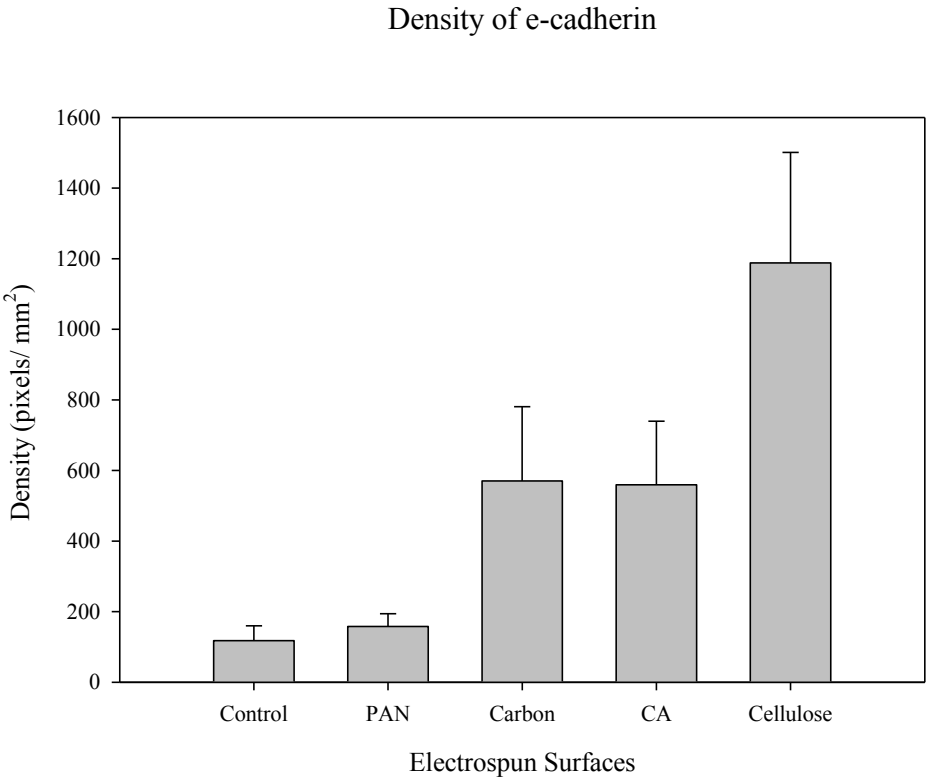


Figure 4-22. Graph showing changes in density of e-cadherin found at cell-cell junctions upon growing on the different electrospun surfaces

Table 4-4

Table showing cadherin analysis

	Control	**PAN**	**Carbon**	**CA**	**Cellulose**
Avg density	117.82	157.73	498.52	559.12	1188.05
Std Dev	42.12	36.45	180.06	180.09	312.95
n	10	10	10	10	10

The density of the adherens junction protein, cadherin was quantified to better understand inter cellular interactions. Control and electrospun PAN surfaces showed a similar quantity of cadherin. Cellulose fibers promoted the best cadherin interaction in MDCKs.

CHAPTER 5

Discussion and Conclusion

This study was performed in an attempt to determine the importance of various characteristics belonging to electrospun nanofibers and their role in determining cell growth when used as a scaffold substrate. The primary characteristic analysis is the size (width) of the electrospun fibers. The results of SEM analysis demonstrated the variance in width from PAN nanofibers to cellulose nanofibers. Data showed that PAN nanofibers had the smallest average size and cellulose nanofibers had the largest average size. It is worth noting that cellulose acetate nanofibers possessed a wider range of fiber sizes, but the average was found to be between that of PAN and carbon nanofibers.

The secondary characteristic of analysis regarding these nanofibrous material is fiber "wettability". The "wettability" refers to the hydrophobicity of nanofibers mat/scaffold. Results of the contact angle assay revealed that the most wettable nanofiber was cellulose while the least wettable fiber was carbon. These characterization techniques allow for a deeper understanding of the fibers from which the scaffold substrate is comprised. The material of fiber along with fiber sizes and wettability are three main points of analysis from which the cellular assays were gauged.

The next notable facet of the analysis is the determination of growth and viability of the MDCKs. This analysis revealed the manner how the cells interact with the scaffold substrate and how the scaffold affects the cellular functions that are needed for growth and viability. The first assay of interest in this regard is a cell counting assay. The results from this assay suggested the greatest growth was found when using PAN nanofiber substrate whereas the least cell growth occurs when the cells were seeded on carbon nanofiber substrate. Statistical analyses of the cell

number through a t-test returned p-values of less than 0.05 for all surfaces except cellulose after a day of growth. After four days of cell growth, p values were all statistically significant except for cellulose acetate and PAN surfaces.

Table 5-1

Table showing statistical analysis of p-values for cell growth

	PAN	**Carbon**	**CA**	**Cellulose**
Day 1	***5.2555E-06***	***0.00091856***	***0.00511091***	***0.16933234***
Day 4	***0.00124036***	***1.008E-08***	***0.42067703***	***0.01371695***

The second area of study with regards to the impact of the nanofibrous substrates was the viability assay. The highest cell viability was observed on glass control substrate followed by the cellulose nanofibrous substrate. Comparatively, the lowest cell viability was found when carbon nanofiber substrate was employed. These assay demonstrated the importance of interactions occurring between the cell and the nanofibrous substrates on which they are grown. There, however, remains one more element of examination through which the impact of material choice for scaffold on cellular function can be observed: cell morphology.

Not only the growth and viability of the cells placed on the scaffold but also the morphology of the cell and the effect thereof on the possible tissue generation capabilities are of importance. There are three main areas of consideration when looking at possible tissue generation: shape of the cell, protein expression of the cell, and size of the cell. The shape of the cell, or “morphology”, is fundamental to optimal functionality of a cell. It enables the cell to optimize its intake of nutrients and expulsion of waste. The resulting data from the assay showed that the cellulose substrate generated the most spherical cell morphologies while the carbon substrate generated spinal cell morphologies. This lends itself directly to the next area of analysis, the protein expression of the cell.

Results of the assay showed that the PAN substrate caused the cells to produce the least amount of cadherin protein while the most cadherin was generated by the cells placed on the cellulose substrate. This leaves the analysis of cell size as related to the substrate material choice. The results of the assay regarding the cell size revealed that the cells grown on the cellulose substrate were the smallest in size, which is closely followed by the carbon substrate as compared to cell size being the largest for those cells grown on control substrate. Electrospun PAN and carbon surfaces showed statistically significant p-values for the cadherin analysis.

Table 5-2

Table indicating statistical p-values of cadherin analysis

	PAN	**Carbon**	**CA**	**Cellulose**
p value	0.484	0.061	0.038	0.007

All of the aforementioned results lead to some interesting notes with regards to the role of various materials and their interaction with cellular functions in the generation of tissue. Material choice was seen to be the greatest factor in affecting cellular morphology. Cell growth was seen to be optimal when the wettability of the surface was between the two extremes. Fiber size was seen to have a direct correlation with the amount of cadherin protein expressed at cell-cell interface.

In the end, the results obtained from this preliminary analyses validated the assumptions regarding the fundamental and inherent characteristics of substrate choice, which greatly affected the functionality of the cells grown on them. Further understanding of these fundamental properties can allow scaffold substrates to be tailored to in such a manner as to promote desired cellular functionality and interactions.

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