DEVELOPMENT AND CHARACTERISATION OF NANO FIBER BASED SKIN EQUIVALENT MODEL

By

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DEVELOPMENT AND CHARACTERISATION OF NANO FIBER BASED SKIN EQUIVALENT MODEL

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Development and Characterization of Nano Fiber Based Skin Equivalent Model

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Abstract

Over the past three decades, significant progress has been made in the area of skin tissue engineering using different types of scaffolds such as non-cell based grafts and cell based grafts. The skin consists of the epidermis, dermis, and hypodermis, the dermis is the thickest layer and so far challenges in skin tissue engineering has been difficulty to restore the dermis. Fibro blasts secrete collagen and elastin which gives mechanical strength and elasticity to the skin which is the main function of the dermis. The aim of this study. is to develop a skin graft model that can mimic the structure and function of the dermis by using Electrospun Nano fiber coating to design polyethylene Glycol Diacrylate(PEGDA) poly e-caprolactone (PCL) scaffolds, these 3 different scaffolds namely (PEGDA) poly e-caprolactone (PCL), Sodium Alginate PCL and Collagen PCL were cultured using fibro blast cells. Analysis of the development and characterization of these fiber based skin grafts was done by checking the architectural integrity by degradation analysis, cell viability through proliferation of cells, cell differentiation, and cell addition. Furthermore, scanning electron microscopy SEM (histology section) of PEGDA PCL, sodium alginate PCL and collagen PCL without cells was used to see the adhesiveness of PCL fibers with the different scaffold. Also, this study involved morphometric analysis through Wettability of PEGDA PCL, Sodium Alginate PCL, and Collagen PCL to see its ability to absorb water. Cytotoxicity of cells showed that cells sticks to the bottom of samples. During this study, it was seen that Collagen PCL and Sodium Alginate PCL showed better characteristics regarding being compatible with the normal skin if used as a skin graft.

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LIST OF ABBREVIATION

PCL	Poly caprolactone
PEGDA	poly ethylene glycol diacrylate
SA	sodium alginate
CG	Collagen
ENF	Electrospun Nanofibers
SEM	Scanning electron microscopy
OCT	Optional cutting Temperature
SBF	Simulated body fluid
HDF	Human dermal fibroblast

CHAPTER 1

INTRODUCTION

1.1 Summary

Scaffolds are structures of artificial or natural materials on which tissue is grown to mimic a biological process outside the body or to replace a diseased or damaged tissue inside the body [39] Hydrogels are water swollen polymeric networks that can swell but do not dissolve in water. Hydrogel scaffolds are engineered to resemble the mechanical properties of skin tissues in the body. Sodium alginate (SA) and collagen (CG), is made of a hydrated polymer gel and was used as a tissue engineering scaffold for soft tissue grafting, in addition, Polyethylene Glycol Diacrylate (PEGDA) was a third skin graft made. SA is very porous; this is due to the fact that it is made from a combination of sodium alginate, calcium chloride and a large volume of water, this porous structure allows cells to grow. CG is the most known protein of the extracellular (ECM) of the skin; this protein stimulates cellular adhesion. Polycaprolactone (PCL) nanofiber matrix is made from an electrospinning process; this matrix was used with SA, CG, and PEGDA to increase mechanical stability and cell adhesion.

1.2 Tissue Engineering

Tissue engineering basically refers to a field of biomaterials development which involves combining scaffolds, cells and biologically active molecules into functional tissues [1]. Tissue engineering aims at improving or restoring damaged tissue and in some cases organs, it works with the fact that cells are the building blocks of tissue and tissues are the basic unit of function in the body. Through the knowledge that cells respond to signals, encounter changes with their environment and organize into tissues and organisms, we were able to make skin grafts with good characteristics that can be used for tissue engineering.

1.3 Human Dermal Fibro Blast (HDF) Cells

A fibroblast is a type of cell that synthesizes the extracellular matrix and collagen [34]. Human Dermal Fibroblast (HDF) is responsible for producing the extracellular matrix forming the connective tissue of the skin and plays an important role during wound healing, they also communicate with each other, and other cell types are thereby playing an important role in regulating skin physiology. Fibroblasts work with keratinocytes to organize a basement membrane which results in a morphologically identifiable structure. It can be harvested from human adult's skin usually from different parts of the skin such as the face, breast, abdomen, and thigh.

1.4 Skin Equivalent Model

The skin is the largest organ in the body, therefore, it's one of the most important organs of the body. It protects the body from physical damages due to assault, the skin also regulates homeostatic and performs sensory functions. Damaged skin is a situation that results in the skin losing its ability to perform these functions, which can lead to various complications such as loss of fluid or infections. Due to the limited understanding of the biological process involved in wound repair, maintenance of skin structure and integrity, also the fact that non-healing wounds present a significant and increasing problem in the health care system more research is being done to see how this problem can be solved. In this research, a skin graft was created using three different models, and its characteristics examined to see compatibility.

1.5 Polycaprolactone (PCL) Electrospun nanofiber (ENF) membrane

The PCL electrospun nanofiber membrane is made by first adding PCL beads to acetone in the proportion of 0.5 grams to 5.0 grams and then it is mixed thoroughly using the sonicating machine after which it is placed in a needle connected to the flow rate control machine. The electrospinning process creates micro to nanometer diameter fibers using an electrostatically driven jet of the above made polymer solution. The fibers produced have

a very good surface to volume ratio which makes it form a good membrane when harvested at right angles to each other, for this research 24 layers were harvested and used to make a sandwich with the hydrogels made. Usually, a single layer of the aligned fibers is collected between two parallel electrodes as shown in figure 1.2, a silicon mold is used to collect the fibers at right angles to each other. When 24 layers of these fibers are collected the hydrogel membranes are placed on it, and another 24 layers of fibers are covered on it to form a three dimensional hydrogel scaffold.

1.6 Hypothesis

The hypothesis of the study is that either adding PCL fibers to PEGDA, SA, CG or adding PCL fibers to SA-CG, or adding PCL fibers to SA and a novel ear tube or CG and a novel ear tube will increase its ability to serve as a skin graft or show that a skin graft added to an ear tube can increase its compatibility with the body. Eventually helping to solve the problem of wound healing of the skin or the possibility of the ear rejecting an ear tube

1.7 Research questions

This thesis addressed the following questions: (1) between SA, CG and PEGDA skin grafts, which one exhibits the best characteristics as a skin graft? (2) Is there any significant difference in the properties of the skin grafts? (3) If CG is combined in equal proportion with SA will this result in a more enhanced performing skin graft? (4) Will there be any form of migration in the fibro blast cells if skin grafts is wrapped around an ear tube? (5) How does the tension ring help create more mechanical strength to the skin grafts made?

1.8 Goals and Objectives

The goal of this research is to make and evaluate the capability of a three-dimensional skin graft as an effective skin equivalent model. The objectives include

determining the in vitro efficacy of SA PCL, CG PCL, PEGDA PCL, SA-CG PCL using human dermal fibroblast cells, checking for cell adhesion, proliferation, and differentiation using standard cell culture and staining assays. Study of cell migration and tension maintaining potential by quantifying proteins present at periphery of cytoplasm of cells on the scaffold.

1.9 Organization of the thesis

The outline of this thesis is simplified in Figure 1.3. There were six chapters. Chapter 1 is the introduction. Chapters 2 demonstrated polyethylene glycol diacrylate (PEGDA) as a combination of PCL fibers cultured with normal human dermal fibroblast (HDF) cells and tested for histology, cell viability, degradation, and wettability. Chapter 3 shows how sodium alginate (SA) and PCL fibers are made into a three dimensional scaffold including the HDF cells, its functionality as a skin graft characterized based on similar conditions as the PEGDA PCL fibers. Chapter 4 involves collagen PCL fibers with HDF cells, where its durability as a skin graft is tested and characterized based on similar conditions as PEGDA in chapter 2. Chapter 5 uses a combination of SA and CG with PCL fibers to form a skin graft to show that a combination of both SA and CG will yield a skin graft of better characteristics. Chapter 6 incorporated a novel ear tube as an addition to the skin grafts made to check for migration of HDF cells towards the ear tube. It shows the compatibility of implants such as ear tubes with the skin graft made.

1.10 Figures

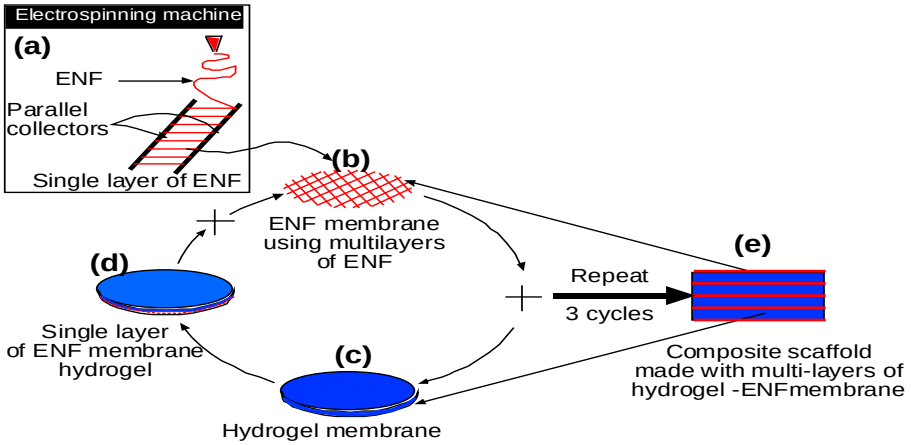


Figure 1.1 Schematic to produce a composite scaffold using electrospun nanofiber (ENF) membranes

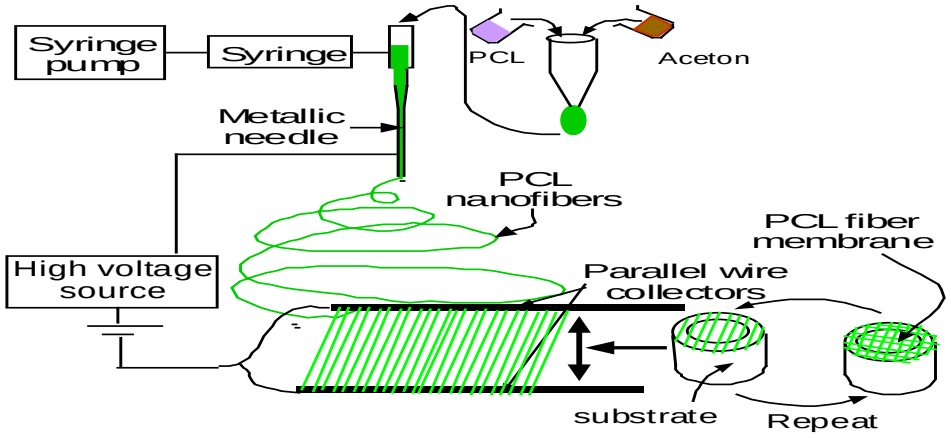


Figure 1.2 Schematic representation of the production of parallel nanofibers and collection of PCL fiber membrane on a round shape silicon mold.

A composite scaffold as a Skin equivalent model							
Aims	**Analysis**	**Subtasks**	**Methods/ Outcomes**	**Sample types**			
				PEGDA -PCL	**SA-PCL**	**Collagen-PCL**	**SA-CG -PCL**
1. Development of scaffold	**Morphometric analysis**	**SEM (histology section)**	**2D internal dimension**				
		Wettablity					
		CT / X ray image	**3D internal dimension**				
		Degradation	**Architectural integrity**				
2. In vitro evaluation	**Biology**	**Cell viability**	**Cytotoxicity**				
			Live/dead assay				
			Proliferation				
			Focal Adhesion				
		Differentiation	**Vimentin**				
			Dapi				
		SA-PCL	**With Tube [Migration Test]**				
		Collagen-PCL	**With Tube [Migration Test]**				

Figure 1.3 Table of the study outline

CHAPTER 2

POLY ETHYLENE GLYCOL DIACRYLATE (PEGDA) POLY CAPROLACTONE (PCL) FIBERS SKIN GRAFT

2.1 Summary

The PEGDA solution is made by mixing 0.3 grams of photo initiator (2,2 dimethyloxy) and one ml of vinly-2-pyrolidinone to form the photo initiator mixture, two ml of PEGDA is mixed with eight ml of 1X phosphate buffer solution to make 20% PEGDA mixture, four micro liters the photo initiator mixture made is then combined with two ml of 20% PEGDA which is cured to form the semi solid PEGDA. The goal of this study is to determine how suitable a PEGDA PCL Nano fiber skin graft can be used as a skin equivalent model for skin tissue engineering regarding restoring maintaining or improving damaged skin if used for ex vitro analysis. Generally, skin tissue engineering involves putting together scaffolds, cells and biologically active molecules to make a functional skin tissue. The development of the PEGDA PCL scaffold was done, and morphometric analysis of scaffold was achieved through SEM histology sections, wettability, and degradation analysis. Also in vitro evaluation was carried out through biological analysis using cell viability, differentiation, proliferation and focal adhesion of PEGDA PCL was accounted for and reasonable conclusions made. Various samples were made to ensure the accuracy of results.

2.2 Background and specification

2.2.1 Introduction

Tissue engineering is a growing field in the global medical industry, as there is an increasing need for improvement of skin healing and wound healing. From previous studies, PEGDA cannot be used as a skin graft for tissue engineering due to its limitations in the area of cell adhesion and migration within the scaffold. Improvements were done by

coating the top and bottom layer of PEGDA with PCL electrospun nanofibers to get rid of this limitations. This study Placed 24 layers of electrospun PCL nanofibers at the top and bottom of PEGDA sample, then added human dermal fibroblast cells to it. The analysis was performed to see if there is cell attachment, proliferation, and differentiation to see how effective PEGDA can be if used as a skin graft. PEGDA is a biocompatible material that supports the growth of cells unto 3D tissues; it has many medical applications especially in the area of tissue engineering. The goal here was to produce a 3D scaffold using PEGDA PCL to see how it can be used as a skin graft for engineering. The size of PEGDA was limited to 0.5 mm because above 1 mm PEGDA begins to have limitations as a three dimensional cell-culture device due to the inability of cells to survive within the scaffold. The biocompatibility of PEGDA is good because it can be absorbed by living tissues over time. PEGDA is a polymer material which is made up of very long molecules with a typical chain of carbon atoms as the backbone. PEGDA Is a photosensitive hydrogel which makes it easy to control its porosity; it is made by careful handling. Many PEGDA hydrogel scaffolds have been created for the Reconstruction of injured tissues, both hard and soft tissues, although the in vivo performances have not yet been reported.

2.2.2 Research questions

This study was conducted based on three research questions: (1) Can PEGDA made with PCL fibers and cultured with HDF cells be developed as a skin graft. (2) Is there a significant difference in the PEGDA scaffold in comparison with other scaffolds such as SA and CG and it’s in vitro evaluation. And (3) how does the SEM histology enable a conclusion to be drawn about PEGDA as a skin graft.

2.2.3 Scope of work

The scope of work for this study was: (1) to develop a three dimensional scaffold using PEGDA and PCL fibers through morphometric analysis such as SEM histology sectioning, wettability, and degradation. (2) to carry out biological evaluation through cell

viability tests such as proliferation, focal adhesion, differentiation, cell attachment, and migration.

2.3 Material and method

The material used were all found in DR Khandaker's lab. For PEGDA PCL, the right quantity of PCL solution was made by ultrasonic mixing of 7.69 wt% of PCL pellets of size approximately 3mm and average Mn 80,000. Acetone, a lab reagent of greater than 99.5%, is mixed with the PCL pellets. The final solution is then poured into a glass syringe pump for fiber production. The PCL fibers are then ejected from the glass syringe through a discharge needle, this needle (23G blunt needle, aluminum make, 1 inches length. Model # BX 25). The needle was charged with 9 kilovolts power supply. Well-made fibers were collected using a wooden block with two parallel electrodes attached to it. 24 layers of fibers were collected manually on acrylic mold through repetition of forwarding and backward motions, each layer of fiber was collected at a right angle to the next layer.

2.3.1 Sample Preparation

Three samples of PEGDA PCL fibers were made, and each sample made into a two layer 24 layer fibers and one layer 0.5mm PEGDA sample in between. This sample was made to represent a skin graft, after which HDF cells were added to make the sample complete. Physical and biological test were conducted as well as cell viability tests. PEGDA sample was cued under UV light for approximately 3 minutes. To cure PEGDA silicon molds were made using acrylic molds to form the shape and a 3;1 mixture of the silicon mold gel, this gel was poured into the acrylic molds after hand mixing thoroughly and placed on a flat surface for 24 hours.

2.3.2 Experiments and analysis

The morphometric analysis which includes the physical characterization of PEGDA PCL scaffold through SEM histological section, here 2-dimensional pictures were taken using the SEM machine; another task was the wettability analysis of samples through immersion in human body fluid solution also called hanks solution. The architectural integrity was examined through degradation by allowing sample stay in hanks solution for 14 days, this process was summarized as the development of the scaffold.

In vitro evaluation involved biological analysis through cell viability tests, these test included differentiation, proliferation, focal adhesion, cytotoxicity and stress fibers stains respectively. During this process HDF cells were cultured with a scaffold for 72 hours in 10ml media solution, after 72 hours 5ml old of media was taken out and replaced with one microliter of EDU nucleotide in each sample, this was left for an hour to activate a differentiation in the cells. Samples were then taken out and fixed; this fixing procedure involves using 4% PFA on each sample for 20 minutes after which samples were rinsed three times with 0.1 molar phosphate buffer solution at 5 minutes interval. 0.05 molar solution was added to samples for 30 minutes, and sample box which is usually a 12 well dish is put on the ice, and then methanol is added for 5 minutes after which we have another three races with 1X PBS solution is done. Nonspecific blocker was used to prevent non-specific background staining for 10 minutes; alpha smooth muscle actin was used to stain samples this stain was left for 48 hours. 7.2% AB goat antimouse Alexa 488 was used to stain samples after refrigerating for 48 hours, click stain is prepared within 10 minutes and added to skin graft for 30 minutes, click stain is added for 15 minutes, and samples cleaned with 1X PBS and mounted on the microscope slide using 80% glycerol. The inverted fluorescent microscope is used to take pictures of skin graft for analysis of results.

For SEM histology sections PEGDA PCL skin graft was made without cells and inserted into optimal cutting temperature fluid (OCT) using tweezers, this tweezer made it easy to push the ear tube to the bottom of the OCT, the procedure was done carefully to prevent any air bubbles from forming in the fluid. The OCT machine was turned on and

allowed to cool down to minus 20 degrees; this usually takes about one hour to cool. After which samples were placed in OCT machine and allowed to freeze. Once there were frozen one sample at a time is placed on the cutting holder and the right cutting size and angle are adjusted to get optimal results. Each slice is collected using microscope glass slide and placed in a file for storage. All cut samples slices are then taken to the SEM lab to capture images

2.3.3 Data analysis

Data were analyzed by physically counting the number of cells that migrated, and attachment to the skin graft proliferated cells, and differentiated cells that were found in each picture. All the best pictures were taken we're used to analyzing how good PEGDA Can be as a skin graft. Also, the comparison was done with standard representation of myofibroblast cells from previous studies to know how good results obtained were. Cell density indicated the concentration of cells per unit area.

Data from physical analysis were plotted to give a clear picture of the process of degradation involved in the produced skin graft.

2.4 Results and Discussion

Figure 2.1a shows the picture taken using an inverted fluorescent microscope; this image displays the cell attachment of PEGDA PCL as indicated in image cells attach to fibers and PEGDA. The image was taken towards the edge of graft due to the high fluorescence of PEGDA. Figure 2.1b is an image for physical characterization, image taken with the SEM machine shows attachment of PCL fibers with PEGDA sample.

Figure 2.2 shows the Vimentin stain (nuclei, differentiation, and proliferation), a close captured cell indicated the presence of proliferation in PEGDA PCL nanofibers skin graft. The effectiveness of displaying the nuclei stain here was also displayed.

Figure 2.3 gives a clear view of just the nuclei/cell attachment of HDF cells to PEGDA PCL skin graft

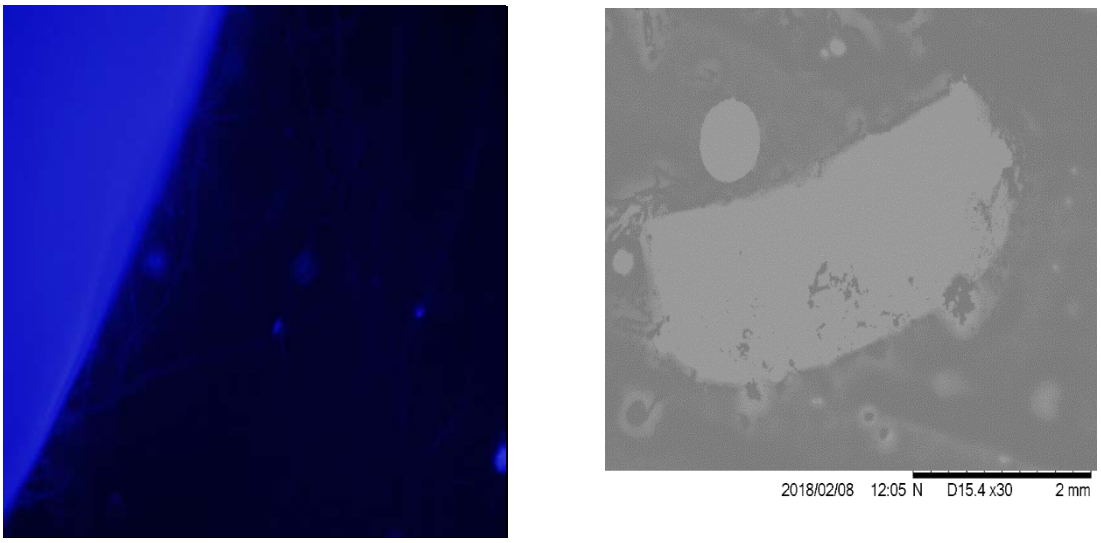
Table 2.1 shows the variation in weight of graft when measured using the lab weight weighing machine; this data shows absorption of fluid and degradation of the sample when placed in fluid for two weeks.

2.5 Conclusion

This study found that PEDGA PCL nanofibers have good qualities regarding migration of HDF cell from media to nanofibers, cells not only migrated but they also attached themselves to the skin graft. Cell biology analysis gave good results showing that Proliferation of cells and cell differentiation was also present. Amount of cells proliferating and differentiating was not as high as expected.

Physical characterization of PEGDA PCL nanofibers skin graft was very successful as graft has good absorption and degradation properties.

2.6 Figures



(a) (b)

Figure 2.1 (a) Cell addition of PEGDA PCL fibers scaffold (b) Histological sectioning of PEGDA PCL fibers scaffold

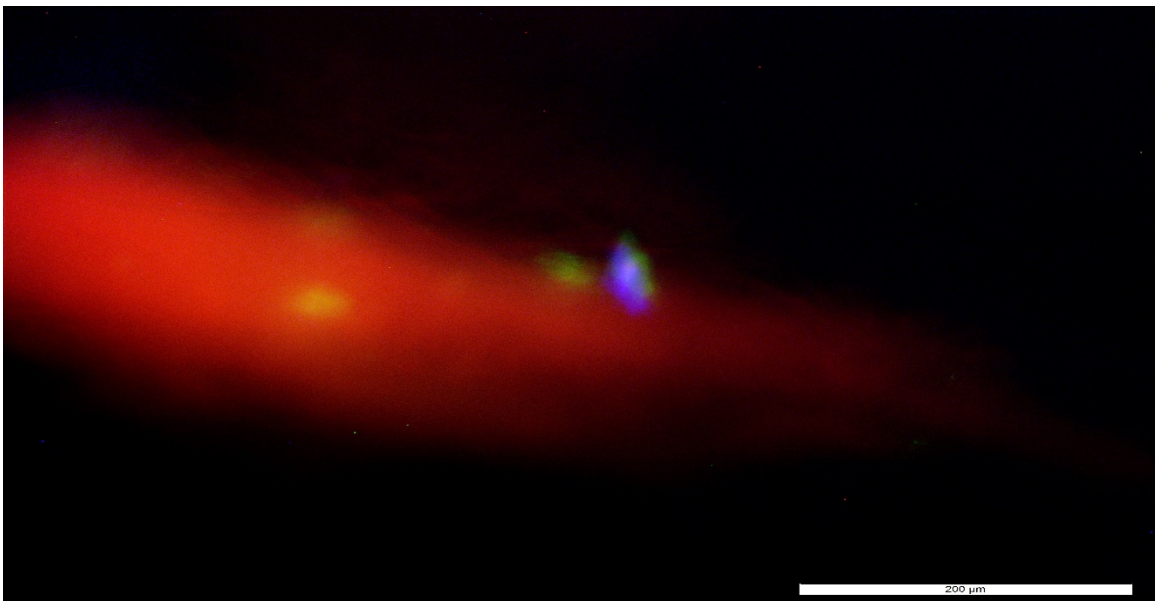


Figure 2.2 Proliferating and differentiating fibro blast cell on PEGDA PCL scaffold

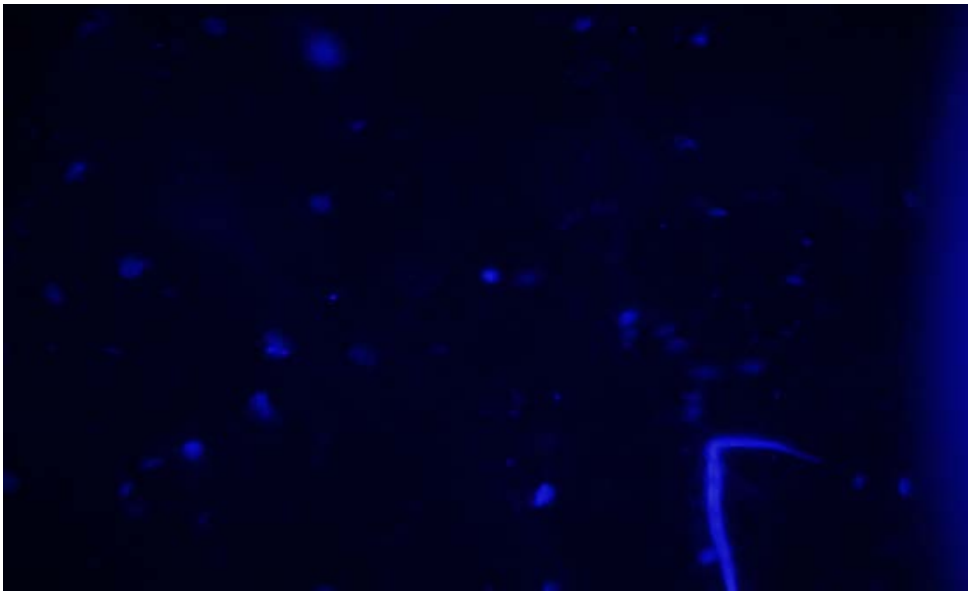


Figure 2.3 PEGDA edge nuclei/cell attachment.

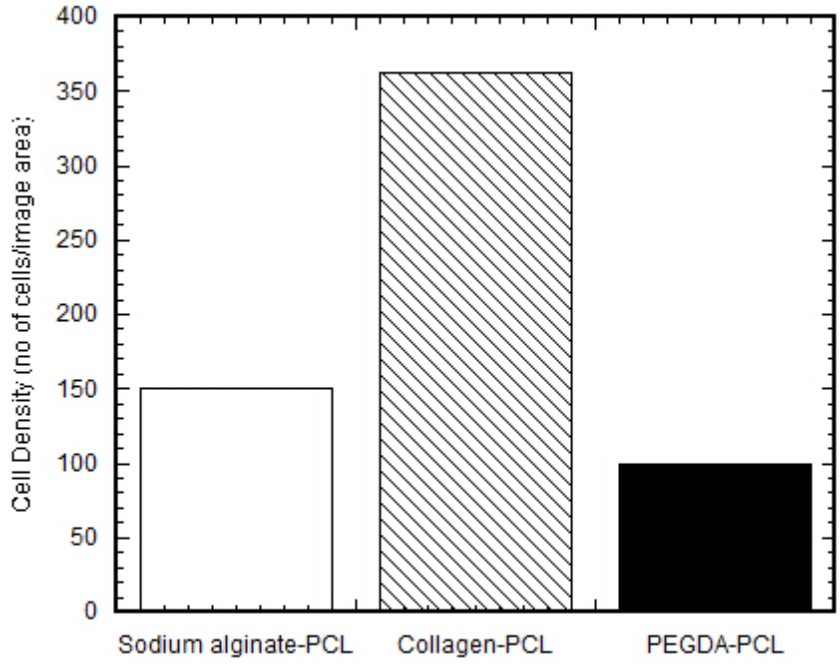


Figure 2.4 proliferation analysis of PEGDA, collagen and sodium alginate.

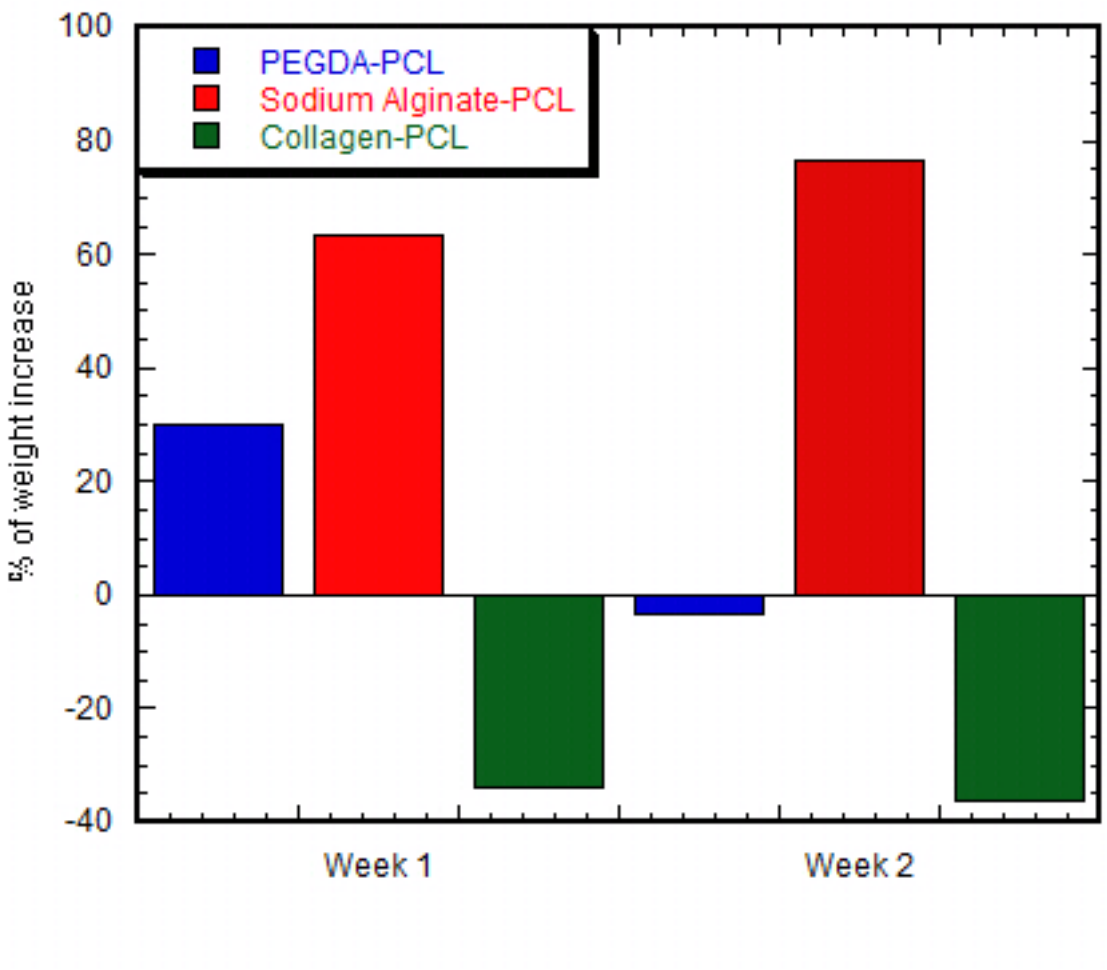


Figure 2.5 Degradation analysis

2.7 Tables

Table 2.1 Degradation analysis of PEGDA PCL skin graft

PEGDA	SAMPLE A	SAMPLE B	SAMPLE C
24 hours	0.1285	0.1125	0.1338
week 1	0.196	0.1526	0.1354
week2	0.128	0.1298	0.1003

CHAPTER 3

SODUIM ALGINATE POLY CAPROLACTONE FIBERS SKIN GRAFT

3.1 Summary

The objective of this study was to determine whether sodium alginate can be used as a skin graft when PCL nanofibers added to sodium alginate hydrogel to enhance its ability to mimic the dermis of the skin. In this study physical analysis through SEM histology is done by sectioning the produced sodium alginate skin graft into different Cut's of various sizes to enable its physical characteristics to be determined. The goal of this study is to determine how suitable an SA PCL Nano fiber skin graft can be used as a skin equivalent model for skin tissue engineering regarding restoring maintaining or improving damaged skin if used for ex vitro analysis.

Also in vitro evaluation was carried out through biological analysis using cell viability, differentiation, proliferation and focal adhesion of SA PCL was accounted for and reasonable conclusions made. Various samples were made to ensure the accuracy of results.

3.2 Background and specification

3.2.1 Introduction

Sodium alginate has been used by other researchers to experiment in different ways; online research shows that not many studies have been done where sodium alginate was used for anything relating to tissue engineering. Due to the gel like properties of sodium alginate, this study includes it as a type of skin graft, the various analysis was done and conclusion made. Methods created in the lab for producing sodium alginates such as freezing and thawing is a unique way to convert a simple mixture of sodium alginate and

calcium chloride into a hydrogel. This scaffold was made with physical and biological characterization of the outcome in mind.

3.2.2 Research questions

This study was conducted based on three research questions: (1) Can Sodium alginate made with PCL fibers and cultured with HDF cells be developed as a skin graft. (2) Is there a significant difference in the SA scaffold in comparison with other scaffolds such as PEGDA and CG and it's in vitro evaluation. And (3) how does the SEM histology enables a conclusion to be drawn about SA as a skin graft.

3.2.3 Scope of work

The scope of work for this study was: (1) to develop a three dimensional scaffold using Sodium alginate and PCL fibers through morphometric analysis such as SEM histology sectioning, wettability, and degradation. (2) To carry out biological evaluation through cell viability tests such as proliferation, focal adhesion, differentiation, cell attachment, and migration.

3.3 Material and method

Sodium alginate powder is weighed to 220 grams and added to 10 ml of water using a 50ml beaker; this is stirred by hand for 3 minutes, the sonicating machine is used to thoroughly mix for 5 minutes with 2 minutes hand mixing intervals, this is done until powder is totally dissolved in water. Calcium chloride is weighed to 110 grams and added to 5ml of water in a 50ml beaker; this is stirred by hand for 3 minutes, the sonicating machine is used to thoroughly mix for 5 minutes with 2 minutes hand mixing intervals, this is done until powder is totally dissolved in water. Both solutions are then mixed together using the sonicating machine.

3.3.1 Sample Preparation

Sodium alginate gel is produced by pipetting 80ul of solution into silicon molds; these silicon molds are then put in the -20 degrees freezer for 2 hours after which they are brought out and allowed to thaw for 1 hour. The right quantity of PCL solution was made by ultrasonic mixing of 7.69 wt% of PCL pellets of size approximately 3mm and average Mn 80,000. Acetone, a lab reagent of greater than 99.5%, is mixed with the PCL pellets. The final solution is then poured into a glass syringe pump for fiber production. The PCL fibers are then ejected from the glass syringe through a discharge needle, this needle (23G blunt needle, aluminum make, 1 inches length. Model # BX 25). The needle was charged with 9 kilovolts power supply. Well-made fibers were collected using a wooden block with two parallel electrodes attached to it. 24 layers of fibers were collected manually on acrylic mold through repetition of forwarding and backward motions, each layer of fiber was collected at a right angle to the next layer. Each 24 layer of fiber is then placed, one on top the SA gel and another below.

3.3.2 Experiments and analysis

Biological tests included focal adhesion and stress fibers test. Three samples of the produced 3-dimensional scaffold were sub cultured for 72 hours, Gabbiani/Hinz fix procedure on skin graft included 0.2% triton in 3% paraformaldehyde solution on samples for 5 minutes, 3% paraformaldehyde for 10 minutes, samples wash with0.1 molar phosphate buffer of PH 7.4 for 30 minutes. Staining for results was by 1 time PBS wash for 5 minutes, block with goat serum for 20 minutes, 100 microliters of vinculin primary anti body was added for one hour at room temperature, rinse with PBS 3 times 5 minutes each time, 100 microliters of secondary anti body which is goat anti mouse alexa 488 plus 1 microliter of Dabbi at room temperature, at this point samples are incubated in the dark as Dabbi is light sensitive. Rise with PBS 3 times 5 minutes each.100 microliters of Rhodamme Phalloidin a stress fiber stain is added to samples for 30 minutes in the dark. Samples are then mounted on the microscope slide using 80% glycerol. Pictures are taken using the inverted fluorescent microscope.

Next experiment was the Vimentin stain; these test included differentiation and proliferation. During this process HDF cells were cultured with a scaffold for 72 hours in 10ml media solution, after 72 hours 5ml old of media was taken out and replaced with one microliter of EDU nucleotide in each sample, this was left for an hour to activate a differentiation in the cells. Samples were then taken out and fixed; this fixing procedure involves using 4% PFA on each sample for 20 minutes after which samples were rinsed three times with 0.1 molar phosphate buffer solution at 5 minutes interval. 0.05 molar solution was added to samples for 30 minutes, and sample box which is usually a 12 well dish is put on the ice, and then methanol is added for 5 minutes after which we have another three races with 1X PBS solution is done. Nonspecific blocker was used to prevent non-specific background staining for 10 minutes; alpha smooth muscle actin was used to stain samples this stain was left for 48 hours. 7.2% AB goat anti mouse Alexa 488 was used to stain samples after refrigerating for 48 hours, click stain is prepared within 10 minutes and added to skin graft for 30 minutes, click stain is added for 15 minutes, and samples cleaned with 1X PBS and mounted on the microscope slide using 80% glycerol. Inverted fluorescent microscope is used to take pictures of skin graft for analysis of results.

A sodium alginate PCL nanofibers skin graft was produced without cells and inserted into optimal cutting temperature fluid (OCT) using tweezers; this tweezer made it easy to push the ear tube to the bottom of the OCT, the procedure was done carefully to prevent any air bubbles from forming in the fluid. The OCT machine was turned on and allowed to cool down to minus 20 degrees; this usually takes about one hour to cool. After which samples were placed in OCT machine and allowed to freeze. Once there were frozen one sample at a time is placed on the cutting holder and the right cutting size and angle are adjusted to get optimal results. Each slice is collected using microscope glass slide and placed in a file for storage. All cut samples slices are then taken to the SEM lab to capture images.

3.3.3 Data analysis

Data were analyzed by physically counting the number of cells that migrated, and attachment to the skin graft proliferated cells, and differentiated cells that were found in each picture. All the best pictures were taken we're used to analyzing how good PEGDA Can be as a skin graft. Also, comparison was done with standard representation of myofibroblast cells from previous studies to know how good results obtained were. Cell density indicated the concentration of cells per unit area.

Data from physical analysis were plotted to give a clear picture of the process of degradation involved in the produced skin graft.

3.4 Results and Discussion

Figure 3.1 shows the Vimentin stain (nuclei, differentiation, and proliferation), a close captured cell indicated the presence of proliferation in the Sodium alginate PCL nanofibers skin graft. The effectiveness of displaying the nuclei stain here was impressive and results satisfactory.

Figure 3.2 is another representation of the Vimentin stain (nuclei, differentiation, and proliferation), a close captured cell indicated the presence of proliferation in the Sodium alginate PCL nanofibers skin graft. The effectiveness of displaying the nuclei stain here was also displayed

Figure 3.3 is an image for physical characterization, image taken with the SEM machine shows attachment of PCL fibers with Sodium alginate sample. This image also tells that PCL fibers totally integrated with SA gel, this is mainly because SA major composition is water.

3.5 Conclusion

Sodium alginate is a good 3-dimensional skin graft. Its physical characteristics especial is the most important as it shows total compatibility with PCL nanofibers. Cell viability was exceptional with little challenges in how good HDF cells can differentiate with SA hydrogel.

3.6 Figures

Figure 3.1 cell proliferation, differentiation, and attachment of sodium alginate PCL nanofibers skin graft. Image 1

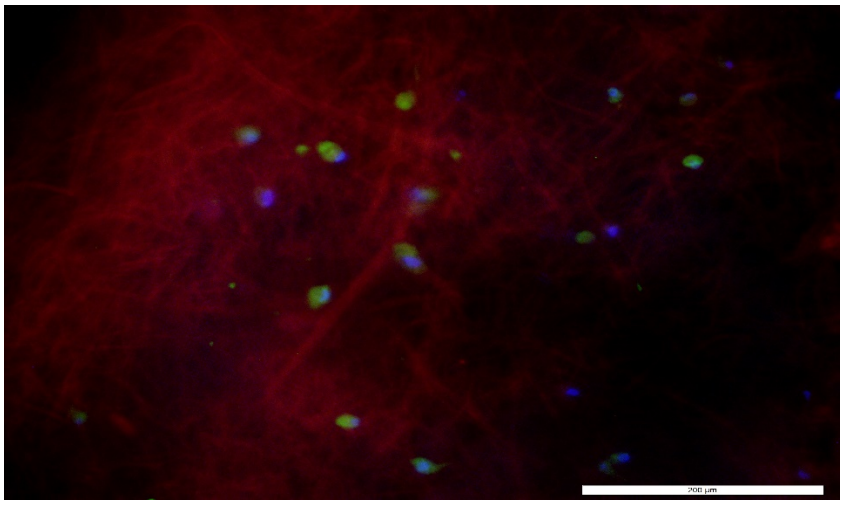


Figure 3.2 cell proliferation, differentiation, and attachment of sodium alginate PCL nanofibers skin graft. Image 2

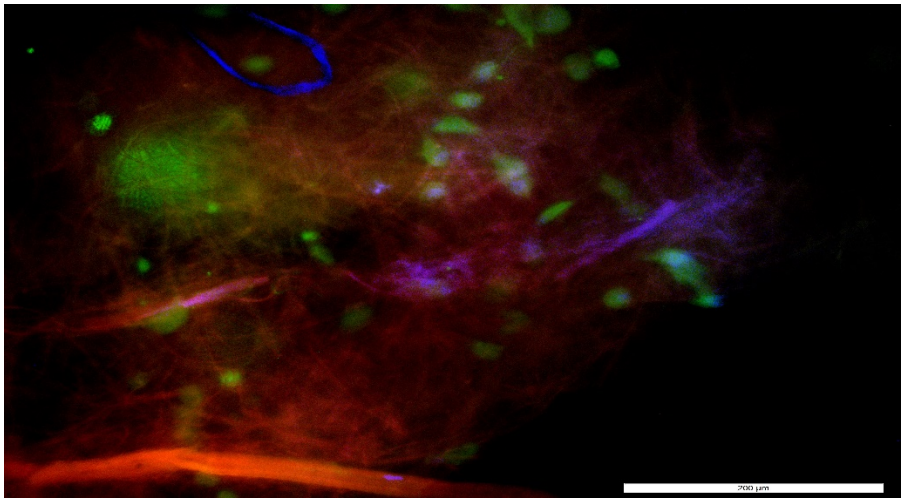


Figure 3.3 SEM histological section of Sodium alginate PCL skin graft

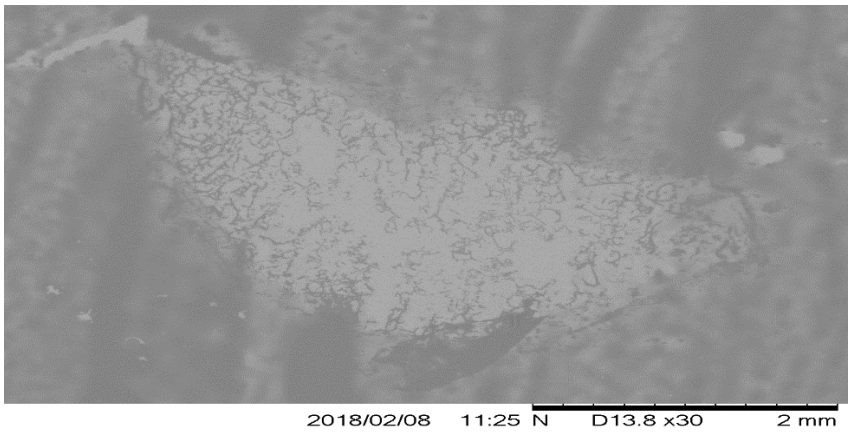
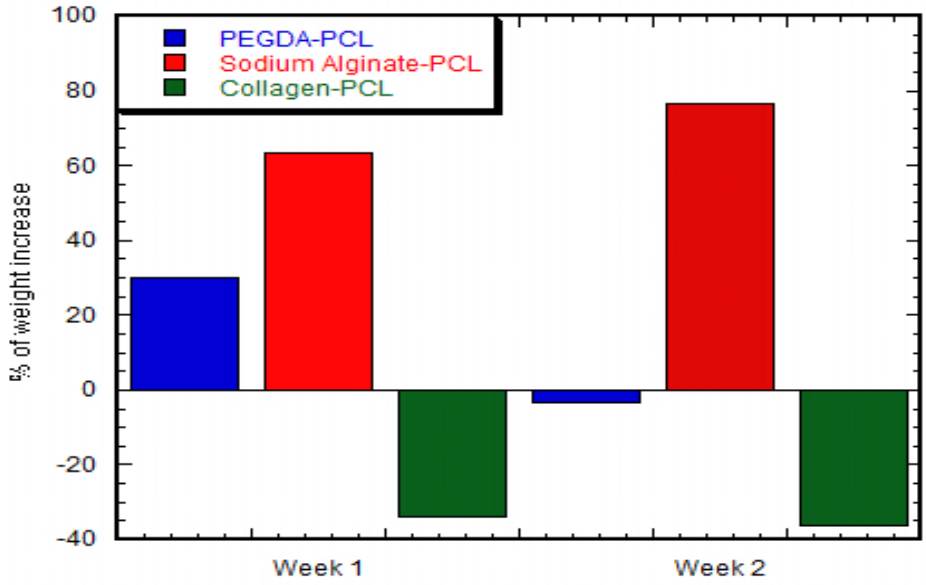


Figure 3.4 Degradation analysis of sodium alginate



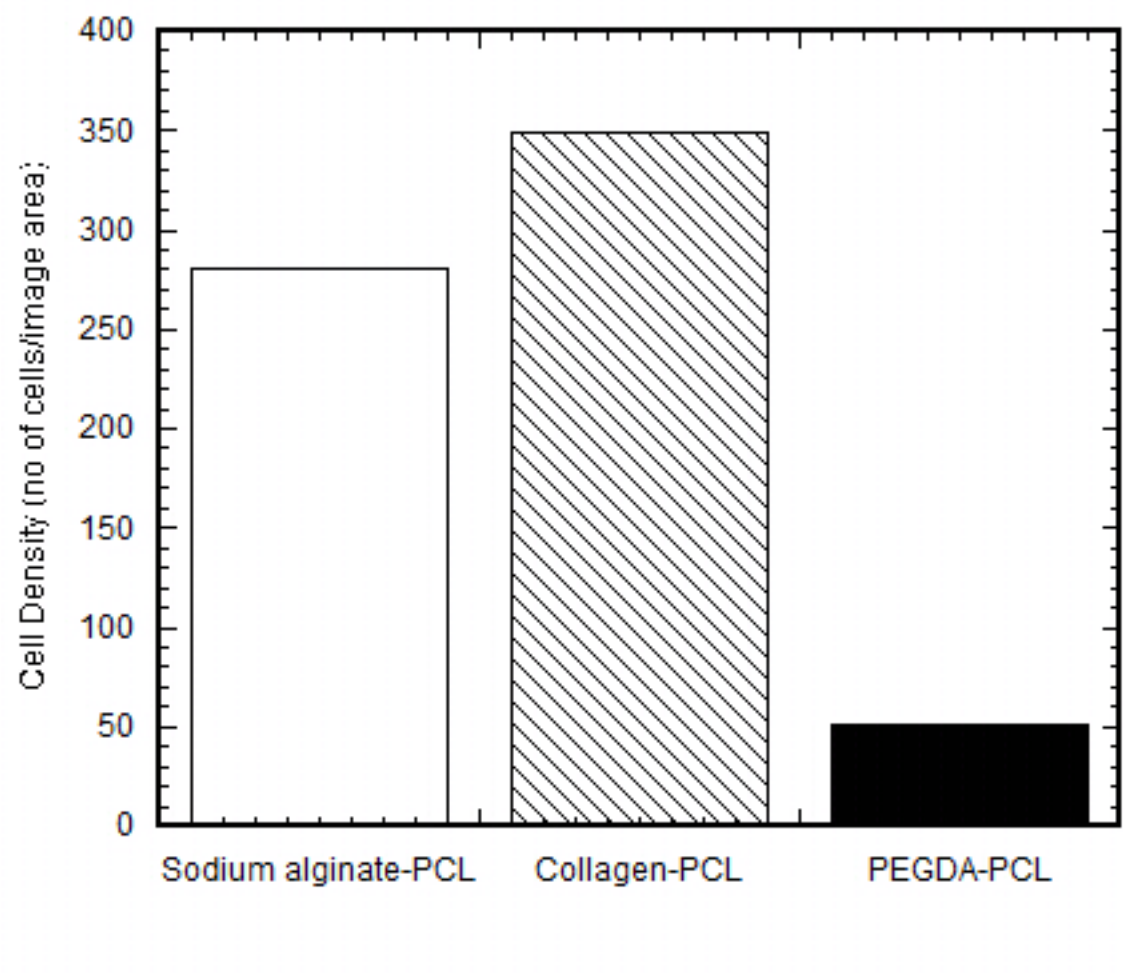


Figure 3.5 differentiation analysis of PEGDA, collagen and sodium alginate.

3.7 Tables

Table 3.1 Degradation analysis of PEGDA PCL skin graft

SADIUM ALGINATE			
24 hours	0.0771	0.0615	0.0728
week 1	0.058	0.1461	0.1293
week 2	0.1091	0.143	0.1135

CHAPTER 4

COLLAGEN POLY CAPROLACTONE FIBERS SKIN GRAFT

4.1 Summary

Collagen is protein made up of amino acids. Collagen hydrogel was made from a combination of collagen concentrate, water, premix, and cells. It is a very good option to consider as a skin graft because the skin itself contains collagen which is the main structural protein found in the extra cellular matrix of the skin. A lot of research has been done about collagen in tissue engineering; this is because promising results were obtained. This thesis research also uses the opportunity to analyze collagen and its characteristics for further results. Morphometric analysis of collagen PCL nanofibers skin graft included scanning electron microscopy of histological sections of skin grafts for physical characterization, wettability, and degradation analysis to determine architectural integrity, in vitro evaluation through biological cell assays such as cell attachment, proliferation, differentiation, focal adhesion, and stress fibers stains respectively

4.2 Background and specification

4.2.1 Introduction

The skin is characterized by the presence of collagen, elastin and extracellular matrix. Collagen is known to secrete extracellular matrix. Skin loss due to skin disease and deep burns has been a problem in the medical field. Desperate measures to restore lost skin has been the driving force of research in the medical field. Tissue engineering has been a promising approach to solve this problem, PCL fibers added to collagen is meant to create a mesh to enhance the effectiveness of collagen as a skin equivalent model. In this thesis, collagen was stained for cell attachment. Differentiation, proliferation, focal adhesion, and stress fibers. This was done because to check for certain characteristics such as conditions necessary for wound healing is important. Cell differentiation implied that cells change

their phenotype from fibroblast to a new phenotype called myofibroblast, this new phenotype is responsible for wound contraction during wound healing. Cell attachment test proves that cell migrate to skin graft and attach themselves thereby making the skin graft its environment to inhabit, this proves that cells will migrate from the body onto the artificial skin graft implanted and make it a new environment which is necessary during wound healing as we want fiber blast cells to always be present at the wound site to the can become myofiberblast and contract the wound. Cell proliferation implies that when cells are present in the granulation tissue at the surface of the wound, they will keep multiplying and producing new parts. Stress fibers are contractile bundles that can be found non muscle cells such as fibroblast; they have an important role in mechanotransduction (the mechanism by which cells convert mechanical stimuli into electrochemical activity) this is responsible for senses such as touch. The presence of dendritic cells shows that cells will be able to communicate with the immune system.

4.2.2 Research questions

This study was conducted based on three research questions: (1) Can Collagen made with PCL fibers and cultured with HDF cells be developed as a skin graft. (2) Is there a significant difference in the Collagen scaffold in comparison with other scaffolds such as SA and PEGDA and it's in vitro evaluation. And (3) how does the SEM histology enable a conclusion to be drawn about Collagen as a skin graft

4.2.3 Scope of work

The scope of work for this study was: (1) to develop a three dimensional scaffold using Collagen and PCL fibers through morphometric analysis such as SEM histology sectioning, wettability, and degradation. (2) To carry out biological evaluation through cell viability tests such as proliferation, focal adhesion, differentiation, cell attachment, and migration.

4.3 Material and method

During the thesis process, the material used included rat tail collagen type 1 of 3.5 ml concentrate, premix, PCL fibers made from acetone and PCL beads using a syringe pump connected to a needle that is powered by a 9 volts power supply. Collagen concentrate preserved in the minus 4 degrees refrigerator is used to make a solution with premix, this premix is basically a combination of different types of salts needed by the collagen to thrive. Sterile water is also a part of the final collagen solution as well as cells.

4.3.1 Sample Preparation

2.6ml of collagen concentrate is taken from a 3.5ml concentrate bottle, 1.45ml of the premix is then added, 0.5ml of cells and media is put next with the mixture then 1.95ml of sterile water is included in the mixture to give a total volume of 6.5ml which can be put on six samples. Then PCL fibers are ejected from the glass syringe through a discharge needle, this needle (23G blunt needle, aluminum make, 1 inches length. Model # BX 25). The needle was charged with 9 kilovolts power supply. Well-made fibers were collected using a wooden block with two parallel electrodes attached to it. 24 layers of fibers were collected manually on acrylic mold through repetition of forwarding and backward motions, each layer of fiber was collected at a right angle to the next layer. One 24 layer of PCL fibers is placed on a tension ring to help collagen create tension with the cells, collagen mix is added the PCL fibers on a tension ring and placed a slide warmer for 20 minutes after which it is placed in the 37 degrees incubator for 72 hours.

4.3.2 Experiments and analysis

The sample staining procedure is started by Biological tests of focal adhesion and stress fibers test. Three samples of the produced 3-dimensional scaffold were sub cultured for 72 hours, Gabbiani/Hinz fix procedure on skin graft included 0.2% triton in 3% paraformaldehyde solution on samples for 5 minutes, 3% paraformaldehyde for 10 minutes, samples wash with0.1 molar phosphate buffer of PH 7.4 for 30 minutes. Staining for results was by 1 time PBS wash for 5 minutes, block with goat serum for 20 minutes, 100

microliters of vinculin primary anti body was added for one hour at room temperature, rinse with PBS 3 times 5 minutes each time, 100 microliters of secondary anti body which is goat anti mouse alexa 488 plus 1 microliter of Dabbi at room temperature, at this point samples are incubated in the dark as Dabbi is light sensitive. Rise with PBS 3 times 5 minutes each.100 microliters of Rhodamme Phalloidin a stress fiber stain is added to samples for 30 minutes in the dark. Samples are then mounted on the microscope slide using 80% glycerol. Pictures are taken using the inverted fluorescent microscope.

Next experiment was the Vimentin stain; these test included differentiation and proliferation. During this process HDF cells were cultured with a scaffold for 72 hours in 10ml media solution, after 72 hours 5ml old of media was taken out and replaced with one microliter of EDU nucleotide in each sample, this was left for an hour to activate a differentiation in the cells. Samples were then taken out and fixed; this fixing procedure involves using 4% PFA on each sample for 20 minutes after wich samples were rinsed three times with 0.1 molar phosphate buffer solution at 5 minutes interval. 0.05 molar solution was added to samples for 30 minutes, and sample box which is usually a 12 well dish is put on the ice, and then methanol is added for 5 minutes after which we have another three races with 1X PBS solution is done. Nonspecific blocker was used to prevent non-specific background staining for 10 minutes; alpha smooth muscle actin was used to stain samples this stain was left for 48 hours. 7.2% AB goat anti mouse Alexa 488 was used to stain samples after refrigerating for 48 hours, click stain is prepared within 10 minutes and added to skin graft for 30 minutes, click stain is added for 15 minutes, and samples cleaned with 1X PBS and mounted on the microscope slide using 80% glycerol. The inverted fluorescent microscope is used to take pictures of skin graft for analysis of results.

4.4 Results and Discussion

Figure 4.1 show collagen cell attachment/nuclei stain, the presence of cells indicated that cells would definitely migrate from the body to an artificial skin graft of collagen and PCL fiber. It also shows that this skin graft creates a stable environment for cells to live.

Figure 4.2 is cells differentiation and proliferation in collagen PCL skin grafts; this gives assurance of cell multiplication, production of new cell parts, the ability of fibroblast cells to change physical characterisics from its normal form to myofibroblast.

Figure 4.3 is an image of stress fibers in the normal human dermal fibrolblast cels used. It indicates that this skin graft produced with collagen and PCL fibers can also restore senses such as touch to the affected wound area.

Figure 4.4 is a representation of dendritic fibroblast cells. This is a good result because not only do we have cell migration and multiplication present but cells can communicate effectively with the immune system.

Table 4.1 a degradation analysis chat of collagen showing how it affected by wear and tear in a liquid environment using human body fluid.

4.5 Conclusion

Collagen PCL fibers is a very good skin graft and had become the best choice amongst all the others used for this project. It did not absorb any fluid during degradation analyses, but its composition gradually decreased. PCL nanofibers was a good way to give more mechanical stability to collagen. Normal collagen without the addition of PCL fibers shows tension in human fibroblast cells when the tension ring is used but in this case where nanofibers were added very little tension was present in fibroblast cells.

4.6 RESULTS

Figure 4.1 collagen cell attachment/nuclei stain

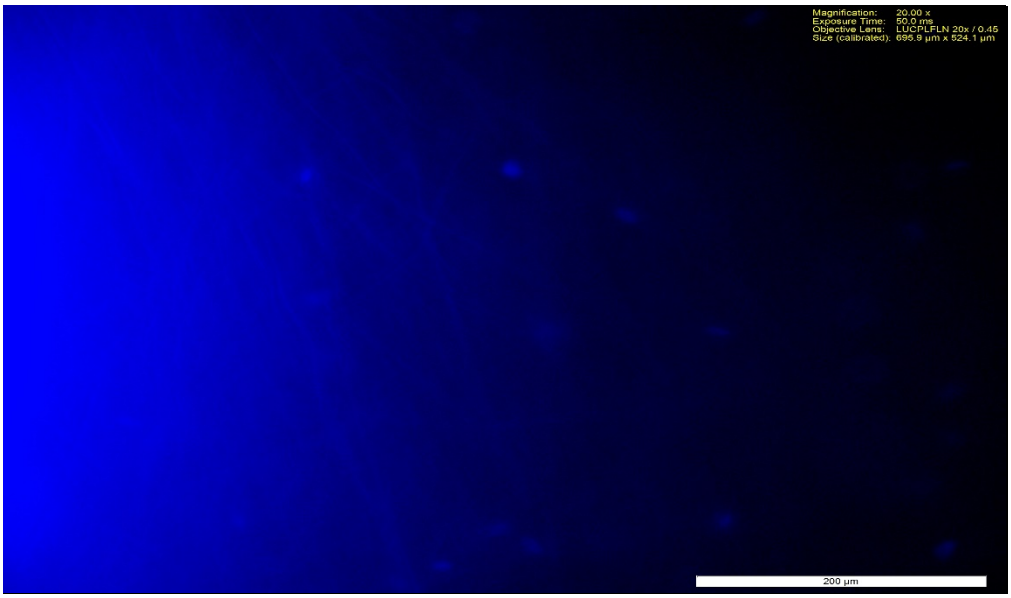


Figure 4.2 cell proliferation, differentiation in collagen PCL nanofiber skin graft

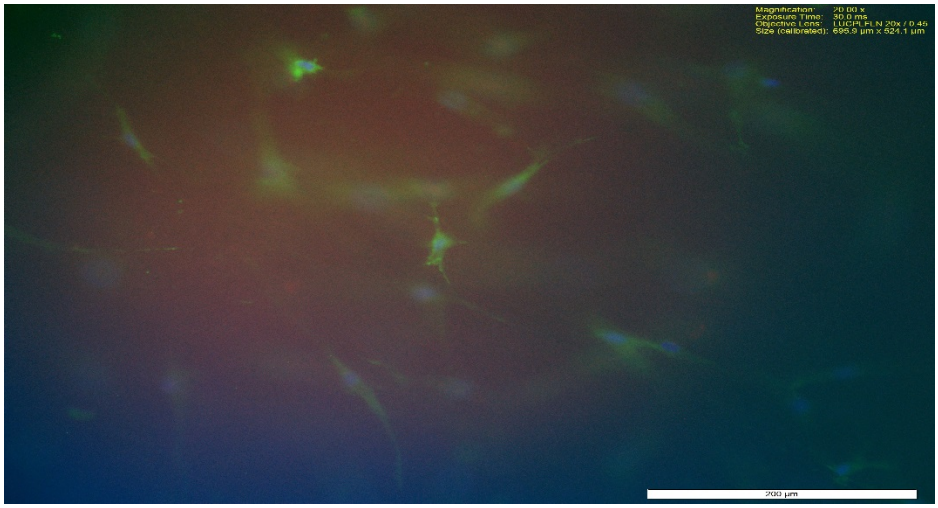


Figure 4.3 stress fibers in human dermal fibroblast cells on collagen PCL skin graft

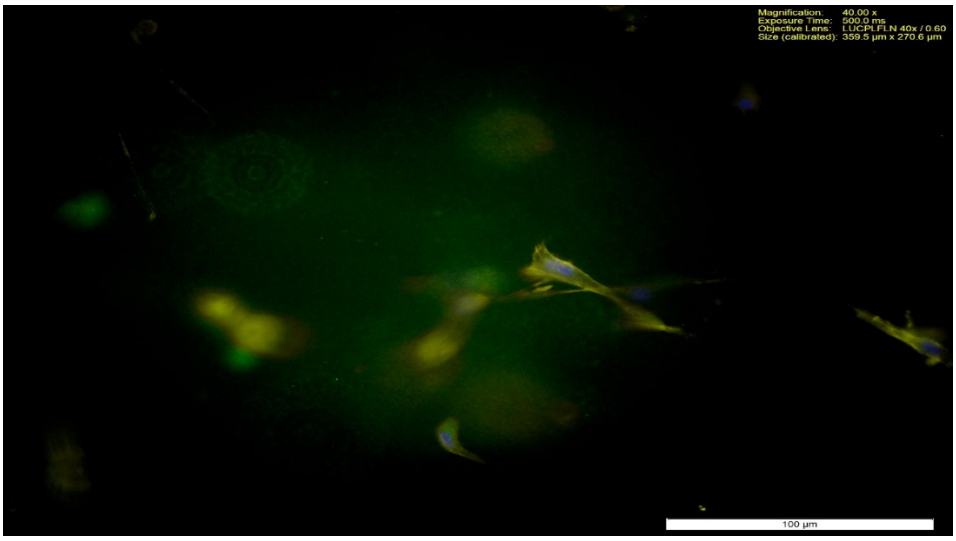


Figure 4.4 dendritic cell extensions in the collagen PCL nanofibers skin graft.

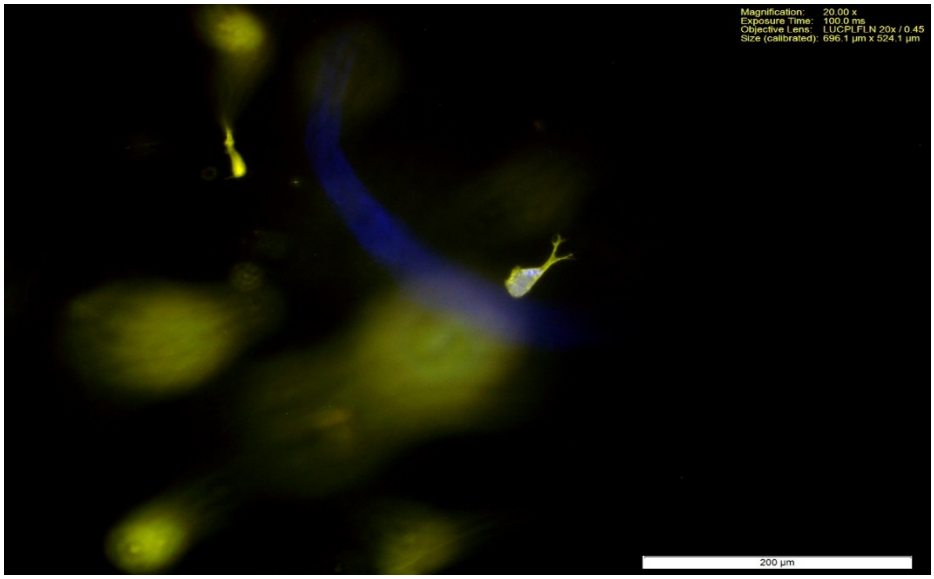


Figure 4.5 degradation analysis of collagen

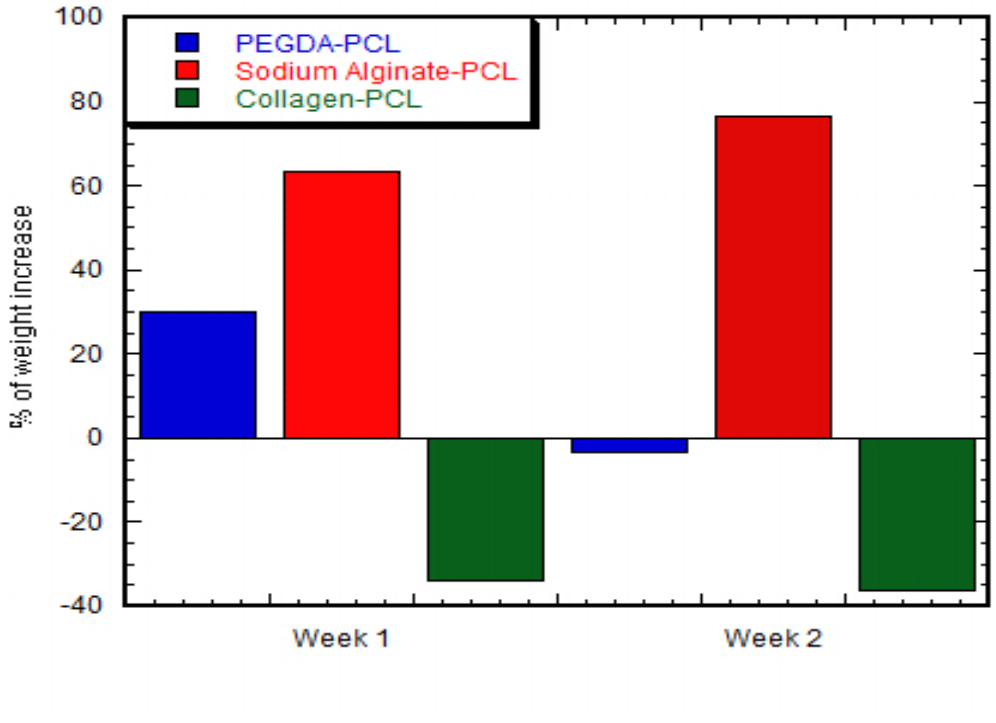
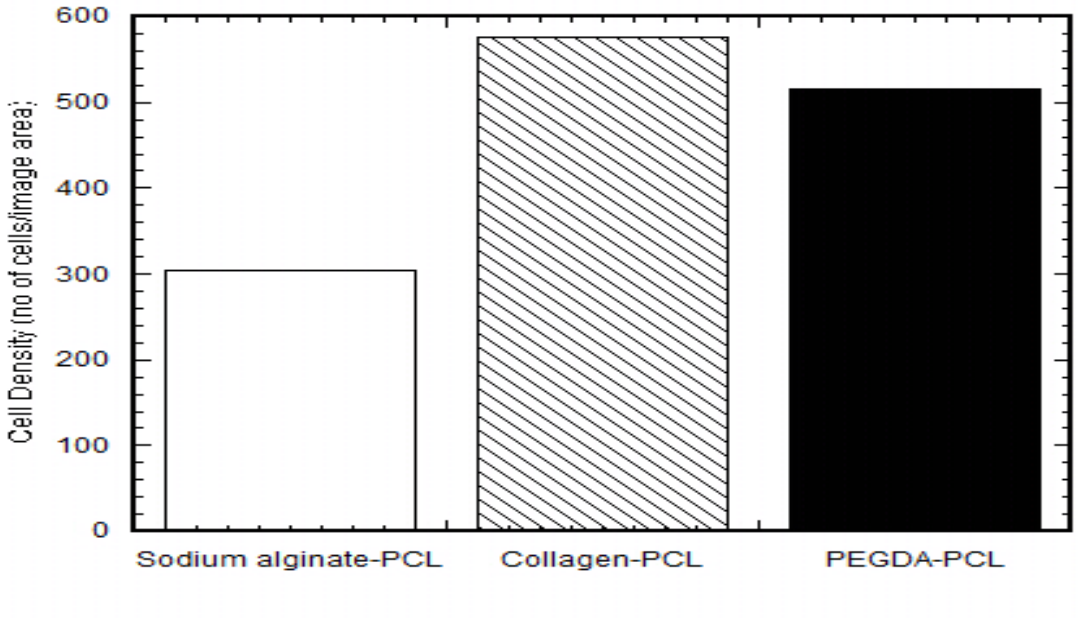


Figure 4.6 cell adhesion analysis of PEGDA, collagen and sodium alginate



4.7 Tables

Table 4.1 degradation analysis table

COLLAGEN			
24 hours	0.4082	0.4586	0.4121
week 1	0.2366	0.3109	0.3001
week 2	0.2175	0.3	0.2987

CHAPTER 5

SODUIM ALGINATE-COLLAGEN POLY CAPROLACTONE FIBERS SKIN GRAFT

5.1 Summary

Out of curiosity and a research mind, an extension was created with a question, what will happen if a new skin graft is created by combining sodium alginate and collagen? Will it give a skin graft of better physical and biological characteristics? Therefore this chapter is about using a 1:1 ratio of collagen and sodium alginate to bring about a more property enhanced skin graft.

5.2 Background and specification

5.2.1 Introduction

The properties of sodium alginate from the study done previously didn't seem as impressive as collagen but there is hope that with the strong characteristics of collagen it will not only stabilize sodium alginate, but it will also lead to improving both of their qualities through weaknesses and strengths of each of them to lead to a wonderful product.

5.2.2 Research questions

This study was conducted based on one research question: (1) can the physiological and biological characteristics of a skin graft made from a combination of sodium alginate and collagen be improved.

5.2.3 Scope of work

The scope of work for this study was: (1) to develop a three dimensional scaffold using sodium alginate-Collagen and PCL fibers. (2) To carry out biological evaluation through cell viability tests such as proliferation, differentiation, cell attachment, and migration.

5.3 Material and method

The material used included rat tail collagen type 1 of 3.5 ml concentrate, premix, PCL fibers made from acetone and PCL beads using a syringe pump connected to a needle that is powered by a 9 volts power supply. Collagen concentrate preserved in the minus 4 degrees refrigerator is used to make a solution with premix, this premix is a combination of different types of salts needed by the collagen to thrive. Sterile water is also a part of the final collagen solution as well as cells. Sodium alginate powder is weighed to 220 grams and added to 10 ml of water using a 50ml beaker, this is stirred by hand for 3 minutes, the sonicating machine is used to thoroughly mix for 5 minutes with 2 minutes hand mixing intervals, and this is done until powder is dissolved in water. Calcium chloride is weighed to 110 grams and added to 5ml of water in a 50ml beaker, this is stirred by hand for 3 minutes, the sonicating machine is used to thoroughly mix for 5 minutes with 2 minutes hand mixing intervals, and this is done until powder is dissolved in water. Both solutions are then mixed using the sonicating machine. A 1:1 ratio of SA and Collagen is added to each well.

5.3.1 Sample Preparation

As written in chapter three and four, the sample preparation was the same. After the procedure in the above chapters was carefully done an equal ration of SA and collagen were added to an already produced 24 layers of PCL fibers.

5.3.2 Experiments and analysis

In vitro evaluation involved biological analysis through cell viability tests, these test included differentiation, proliferation, and cytotoxicity stains respectively. During this process HDF cells were cultured with a scaffold for 72 hours in 10ml media solution, after 72 hours 5ml old of media was taken out and replaced with one microliter of EDU nucleotide in each sample, this was left for an hour to activate a differentiation in the cells. Samples were then taken out and fixed; this fixing procedure involves using 4% PFA on

each sample for 20 minutes after which samples were rinsed three times with 0.1 molar phosphate buffer solution at 5 minutes interval. 0.05 molar solution was added to samples for 30 minutes, and sample box which is usually a 12 well dish is put on the ice, and then methanol is added for 5 minutes after which we have another three races with 1X PBS solution is done. Nonspecific blocker was used to prevent non-specific background staining for 10 minutes; alpha smooth muscle actin was used to stain samples this stain was left for 48 hours. 7.2% AB goat antimouse Alexa 488 was used to stain samples after refrigerating for 48 hours, click stain is prepared within 10 minutes and added to skin graft for 30 minutes, click stain is added for 15 minutes, and samples cleaned with 1X PBS and mounted on the microscope slide using 80% glycerol. The inverted fluorescent microscope is used to take pictures of skin graft for analysis of results.

5.3.3 Results and Discussion

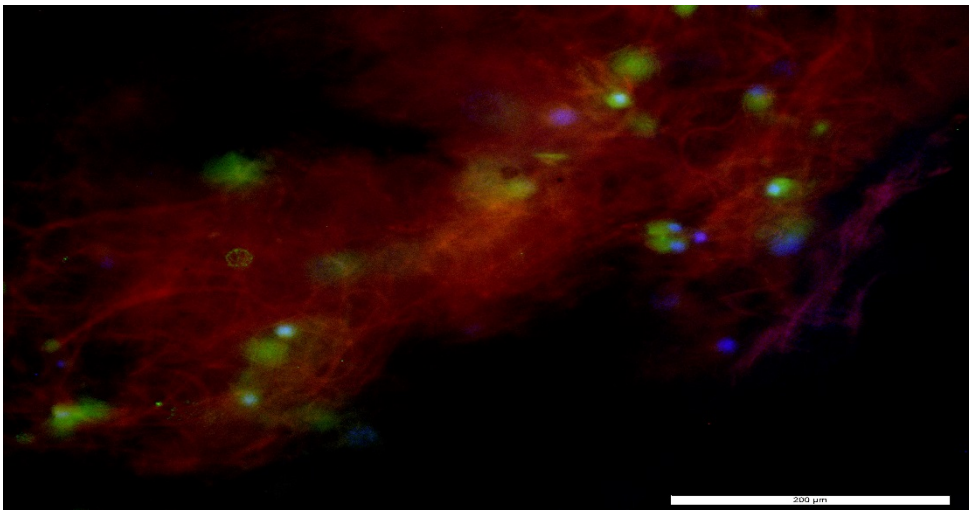
Figure 5.1 shows sodium alginate collagen cell attachment/nuclei stain, the presence of cells indicated that cells would migrate from the body to an artificial skin graft of collagen and PCL fiber. It also shows that this skin graft creates a stable environment for cells to live. Cells differentiating and proliferating in sodium alginate collagen PCL skin grafts gives the assurance of cell multiplication, production of new cell parts, the ability of fibroblast cells to change physical characteristics from its normal form to myofibroblast.

5.4 Conclusion

From the experiment, it was observed that combining sodium alginate and collagen together resulted in the characteristics of collagen being impeded by those of sodium alginate. Cells appeared to be rounded and not extended to indicate a wide range of differentiation. Cell attachment and nuclei results were promising and good. Sodium alginate and collagen can be used as a skin graft with more additive to create a good environment for cells to thrive.

5.5 Figures

Figure 5.1 cell proliferation, differentiation and attachment in sodium alginate collagen PCL nanofiber skin graft



CHAPTER 6

COMPATIBILITY OF COLLAGEN PCL FIBERS WITH A NOVEL EAR TUBE

6.1 Summary

This research focuses on ear tubes that can be used on infants to relieve them of the problem of ear infections. This chapter talks about how our PCL nanofibers were manually wrapped around the ear tube; the tests carried out on the ear tube, data achieved with results and conclusions made. It was an interesting procedure as this ear tubes are so tiny and made out of silicon, this made handling and wrapping of PCL nanofibers quite a challenge.

6.2 Background and specification

6.2.1 Introduction

Ear tubes are very small or tiny cylinders that are inserted into a child's ear to enable the flow of air in and out of the ear; it helps prevent negative pressure in the ear as well as the fluid buildup. The need for ear tube implants had been persistent as a lot of kids are prone to have an ear infection before they reach the age of 5. Therefor when considering the type of body implant to use for compatibility teat with our skin graft, a novel ear tube came to mind. Ear tubes are usually administered when fluid buildup in the ear occurs severally in a child. Ear tubes are very useful; this is because not only do the cure ear infections, but they also prevent long term hearing loss in children. Ear infections in children happen to occur when fluid gathers in the middle ear and cannot drain because the Eustachian tubes get filled up with mucus which aids bacteria to grow, and this can lead to infection. This condition is most likely to happen in situations like when the child has a cold. Ear tube insertion can only be done through surgery which usually does not take more than 20 minutes. On the average ear tubes are inserted in children between the ages of 1 to 3 years. The amazing thing about using ear tubes to cure infection is that parents have to worry only about one surgery as ear tubes extrude themselves (forced out) as the child

grows older. Ear tubes are beneficial not only for curing ear infections but also for improving speech, correcting problems with balance, enhancing good sleep, and decreasing pain in the ear. When ear tubes are inserted and happen not to fall out as expected, they have to be surgically removed and if infections persist they will have to do another surgery to get the ear tubes back in the ear. This research wants to see it the ear canal skin tissue can be replaced by our skin graft the case where some skin is lost in the ear canal.

6.2.2 Research questions

This study was conducted based on one research question: (1) can our skin graft be compatible with the ear tube? If yes then it has the potential to replace the ear canal skin tissue.

6.2.3 Scope of work

The scope of work for this study was: (1) develop a skin graft around an ear tube by adding PCL nanofibers to a novel ear tube and following the procedure for making our best skin graft. (2) To carry out scanning electron microscopy images of ear tubes for physical attachment of ear tubes with the skin graft.

6.3 Material and method

The material used included silicon made ear tubes. PCL, acetone, and collagen. Tweezers were used to hold ear tubes below the parallel electrodes where the PCL nanofibers where collected, secondly nanofibers were attached by the circular motion of the tweezers holding the ear tubes in position; this was done continuously until ear tube was total covered in PCL fibers and its blue color no longer visible. Collagen mix and cells were the added to complete the sample.

6.3.1 Sample Preparation

Sample preparation was done by manually wrapping PCCL nanofibers around the ear tube. Collagen mixture prepared by 2.6ml of collagen concentrate taken from a 3.5ml

concentrate bottle, 1.45ml of the premix is then added, 0.5ml of cells and media is put next with the mixture then 1.95ml of sterile water is included in the mixture to give a total volume of 6.5ml which can be put on six samples. Then PCL fibers are ejected from the glass syringe through a discharge needle, this needle (23G blunt needle, aluminum make, 1 inches length. Model # BX 25). The needle was charged with 9 kilovolts power supply. Well-made fibers were collected using a wooden block with two parallel electrodes attached to it. 24 layers of fibers were collected manually on acrylic mold through repetition of forwarding and backward motions, each layer of fiber was collected at a right angle to the next layer. One 24 layer of PCL fibers is placed on a tension ring to help collagen create tension between the cells, collagen mix is added the PCL fibers on a tension ring and the ear tube inserted in the middle of the skin graft and placed a slide warmer for 20 minutes after which it is placed in the 37 degrees incubator for 72 hours.

6.3.2 Experiments and analysis

Ear tube was taken out of hydrogel solution and inserted into optimal cutting temperature fluid (OCT) using tweezers; this tweezer made it easy to push the ear tube to the bottom of the OCT, the procedure was done carefully to prevent any air bubbles from forming in the fluid. The OCT machine was turned on and allowed to cool down to minus 20 degrees; this usually takes about one hour to cool. After which samples were placed in OCT machine and allowed to freeze. Once there were frozen one sample at a time is placed on the cutting holder and the right cutting size and angle are adjusted to get optimal results. Each slice is collected using microscope glass slide and placed in a file for storage. All cut samples slices are then taken to the SEM lab to capture images.

6.4 Results and Discussion

Figure 6.1: gives an idea of how the ear tube looks when inserted into the ear during surgery, it is very close to the ear canal skin, and it’s of utmost importance that it is compatible with the skin.

Figure 6.2, 6.3 and 6.4: compatibility of ear tube with Collagen PCL nanofibers skin graft was achieved and is illustrated in this SEM histological sectioning.

6.5 Conclusion

Our novel ear tube is compatible with the Collagen PCL nanofibers skin graft. More care in the handling of ear tube is required as ear tube is fragile and even more delicate when frozen below minus 20 degrees Celsius.

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6.6 Figures

Figure 6.1 an illustration of a novel ear tube in the middle ear.

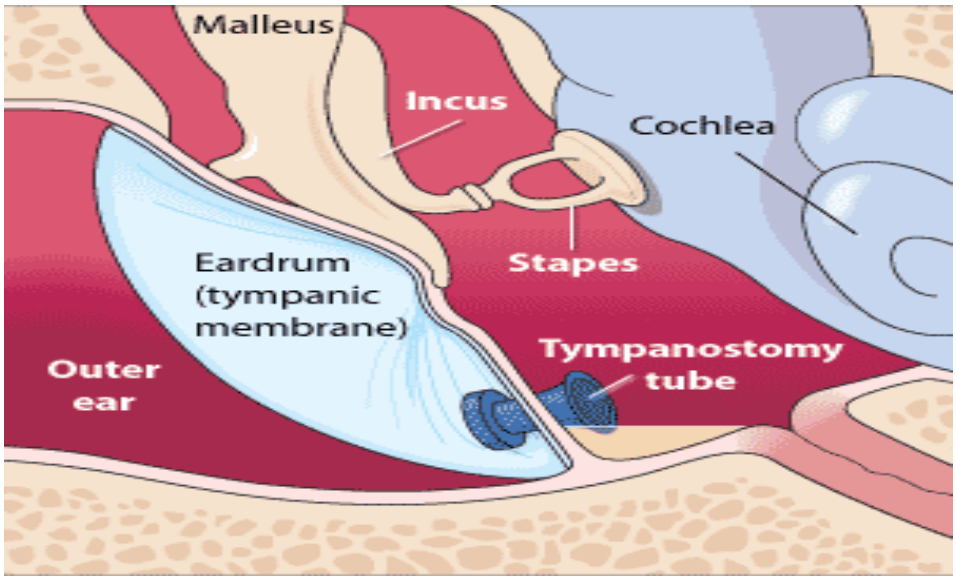


Figure 6.2 Scanning electron microscopy image of the ear tube with a skin graft (40x magnification)

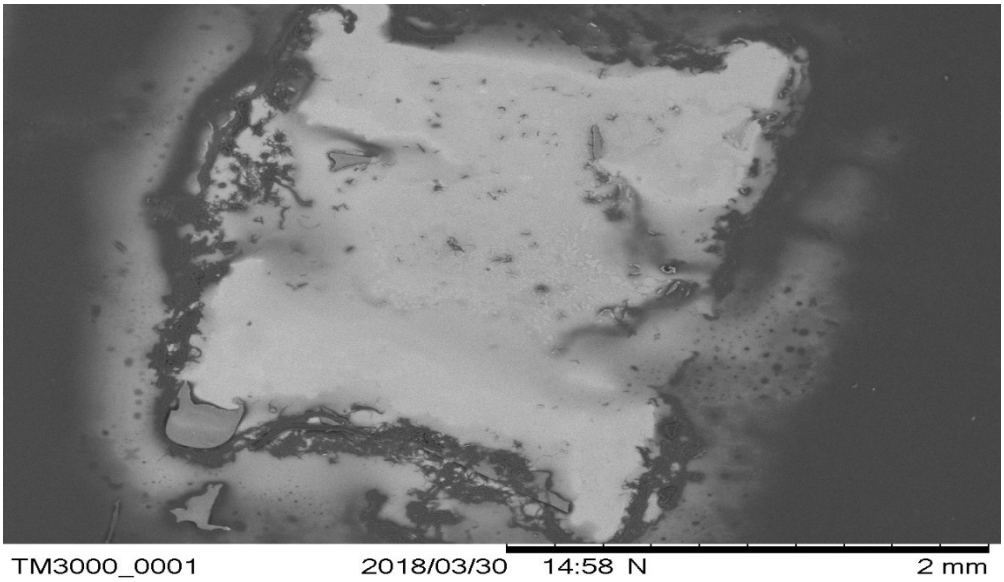


Figure 6.3 smaller section SEM image of ear tube

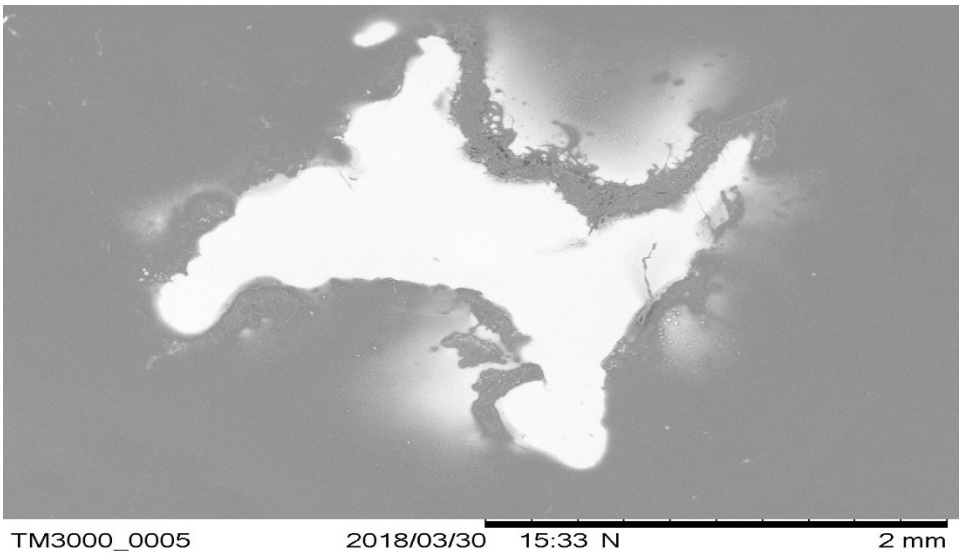
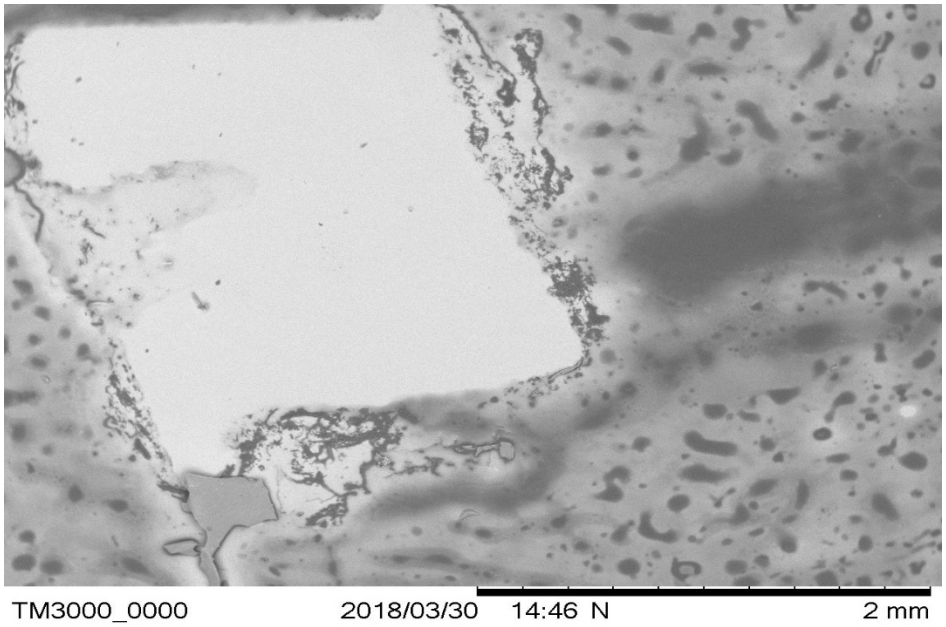


Figure 6.4 SEM histology sectioning



CHAPTER 7

CONCLUSION AND FUTURE WORKS

7.1 Conclusions

From goal (1) our conclusions about PEGDA PCL nanofibers scaffold was, This study found that PEDGA PCL nanofibers have good qualities regarding migration of HDF cell from media to nanofibers, cells not only migrated but they also attached themselves to the skin graft. Cell biology analysis gave good results showing that Proliferation of cells and cell differentiation was also present. Amount of cells proliferating and differentiating was not as high as expected. Physical characterization of PEGDA PCL nanofibers skin graft was very successful as graft has good absorption and degradation properties. Goal (2) concluded that Sodium alginate is a good 3-dimensional skin graft. Its physical characteristics especial is the most important as it shows total compatibility with PCL nanofibers. Cell viability was exceptional with little challenges in how good HDF cells can differentiate with SA hydrogel. Goal (3) our collagen 3-dimensional skin graft PCL fibers is a very good skin graft and had become the best choice amongst all the others used for this project. It did not absorb any fluid during degradation analyses, but its composition gradually decreased. PCL nanofibers was a good way to give more mechanical stability to collagen. Normal collagen without the addition of PCL fibers shows tension in human fibroblast cells when the tension ring is used but in this case where nanofibers were added very little tension was present in fibroblast cells. Goal (4) from the experiment it was observed that combining sodium alginate and collagen together resulted in the characteristics of collagen being impeded by those of sodium alginate. Cells appeared to be rounded and not extended to indicate a wide range of differentiation. Cell attachment and nuclei results were promising and good. Sodium alginate and collagen can be used as a skin graft with more additive to create a good environment for cells to thrive. Goal (5) Our novel ear tube is compatible with the Collagen PCL nanofibers skin graft. More care in the handling of ear tube is required as ear tube is fragile and even more delicate when frozen below minus 20 degrees Celsius.

7.2 Future works

(1). Calcium alginate has been broadly used by other researchers to test its ability to be a good 3-dimensional model. This research proposes that calcium alginate should be used for more studies in the future. (2) From online research and advice from Dr. Vaughan DP 139 cells produce the best focal adhesion stains he also suggested that rabbit tendon cells can be attempted for better focal adhesion stains. (3) In vivo studies should be done to verify collagen PCL nanofibers as a good skin equivalent model for tissue engineering. (4) Further studies on the ear tube can be done with better results if fibers can be wrapped professionally.

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