Universidade do Minho
Escola de Engenharia

Lucas Bernardes Naves

Development of New Alternative Approach for the Treatment of Melanoma Skin Cancer

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Professor Doutor Luis Manuel Guimarães Almeida
e do
Professor Doutor Seeram Ramakrishna

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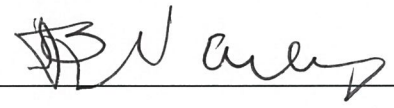
Signature: 

Dedication

This thesis work is dedicated to my lovely mom, Eliane Bernardes, a strong and gentle soul who taught me the values to become honorable man and has been a constant source of encouragement and support during this journey.

Esta tese é dedicada à minha adorável mãe, Eliane Bernardes, uma alma forte e gentil que me ensinou os valores para se tornar um homem honrado e tem sido uma fonte constante de encorajamento e apoio durante esta jornada.

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Abstract

In this study, we present a potential alternative approach for the treatment of melanoma skin cancer and skin tissue regeneration. A comparison of polycaprolactone (PCL) and polycaprolactone blended with linear (LPEI) and branched polyethylenimine (BPEI). This research presents the biocompatibility and feasibility of PCL, PCL loaded with LPEI and PCL loaded with BPEI in different concentrations, producing electrospun scaffolds. SEM images show that the nanofibers developed between 271 to 419 nm. Contact angle assay demonstrated high hydrophobicity for all mats, which could be overcome by surface modification, namely, plasma treatment, ameliorating the hydrophilicity of the mats, providing excellent cells adhesion to the scaffolds surface. We demonstrate the biocompatibility of the scaffolds developed by electrospinning techniques, followed by *in vitro* tests with Human Dermal Fibroblasts (HDFs) and murine melanoma cells (B16), by using MTT assay 3-(4,5- dimethylthizol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium to determinate the biocompatibility with all cells, and confocal images to give us insights of cell morphology (nucleus and cellular membrane). Sirius red collagen assay was performed for HDFs to give the collagen release profile after 6 days of incubation, and the possibility of the mats to help in skin regeneration process by forming extra cellular matrix (ECM). The CFMDA dye suggested no cytotoxicity for HDFs to monitor the morphology of live cells. The results have shown that all the scaffolds developed have good cell adhesion and proliferation properties. We could also observe high cytotoxicity for B16 melanoma cells for PCL_2BPEI scaffolds. This primary *in vitro* study suggests that the mats developed may increase the skin regeneration process and at the same time promote apoptosis of melanoma cells.

Keywords: Electrospinning, drug delivery, melanoma, scaffolds, tissue engineering.

Resumo

Neste estudo, apresentamos uma abordagem alternativa como potencial tratamento para o câncer de pele melanoma e regeneração do tecido da pele. Uma comparação de policaprolactona (PCL) e policaprolactona misturada com polietilenoimina linear (LPEI) e ramificada (BPEI). Esta pesquisa apresenta a biocompatibilidade e viabilidade de PCL, PCL carregado com LPEI e PCL carregado com BPEI em diferentes concentrações, produzindo estruturas de suporte eletrofuncionais. Imagens de SEM mostram que as nanofibras se desenvolveram entre 271 e 419 nm. O ensaio de ângulo de contato demonstrou alta hidrofobicidade para todas as nanofibras, o que poderia ser superado por modificação de superfície, ou seja, tratamento com plasma, melhorando a hidrofilicidade das nanofibras, proporcionando excelente adesão celular à superfície das estruturas de suporte. Demonstramos a biocompatibilidade das estruturas de suporte desenvolvidos por técnicas de eletrofiação, seguidos por testes *in vitro* com fibroblastos dérmicos humanos (HDFs) e células de melanoma murino (B16), usando o ensaio MTS 3- (4,5-dimetiltiazol-2-il) -5- (3-carboximetoxifenil) -2 (4- sulfofenil) -2H-tetrólio para determinar a biocompatibilidade com todas as células, e imagens confocais para dar como insights da morfologia celular (núcleo e membrana celular). O ensaio de colagénio vermelho Sirius foi realizado para HDFs para dar o perfil de liberação de colagénio após 6 dias de incubação, e a possibilidade das estruturas de suporte ajudarem no processo de regeneração da pele através da formação de matriz extracelular (ECM). O corante Diacetato de CFMDA-5-clorometilfluorescea sugere não citotoxicidade das células HDFs monitoradas. Os resultados mostraram que todas as estruturas de suporte desenvolveram boas propriedades de adesão e proliferação celular. Também pudemos observar alta citotoxicidade para células de melanoma B16 para estrutura de suporte PCL_2BPEI. Este estudo *in vitro* sugere que as estruturas de suporte desenvolvidas podem aumentar o processo de regeneração da pele e ao mesmo tempo promover a apoptose das células do melanoma.

Palavras-chave: Eletrofiação, liberação de drogas, melanoma, estrutura de suporte, engenharia de tecidos.

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List of Acronyms

AFM	Atomic Force Microscopy
ASTM	American Society for Testing and Materials
BCCs	Basal Cell Carcinoma
BET	Gas absorption- Brunauer- Emmett- Teller Method
BJH	Barrett- Joyner- Halenda Analysis
BPEI	Branched Polyethyleneimine
BSE	Backscattered Electrons
CAFs	Cancer- Associated Fibroblasts
CE	Electrodessication
CMFDA	5-Chloromethylfluorescein Diacetate
CMs	Cutaneous Malignant Melanoma
CNT	Carbon Nanotubes
CO_2	Carbon Dioxide
CTLA	4- Cytotoxic T Lymphocyte Antigen 4
CUR	Curcumin
DDS	Drug Delivery System
DFB	Dermal Fibroblasts
DI	Deionized Water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOX	Doxorubicin
DTIC	Dacarbazine
EC	Endothelial Cells
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetra- Acetic Acid
EGF	Epithelial Growth Factor
EU	European Union
FBS	Fetal Bovine Serum
FDA	Food and Drugs Administration
FSP1	Fibroblast- Specific Protein 1
FT-IR	Fourier- Transform Infrared

G	Gas
GAGs	Glycosaminoglycans
h	Hour
HA	Hyaluronic Acid
HAP	Hydroxyapatite
HDF	Human Dermal Fibroblast
HIF	Hypoxia Inducible Factors
HMDS	Hexamethyl Disilazane
HTS	High Through Screening
L	Liquid
LG	Liquid-Gas
LIP-CLOD	Liposome- Encapsulate Clodronate
LPEI	Linear Polyethyleneimine
LPS	Lipopolysaccharide
IGF-1	Insulin Growth Factor-1
M	Microphage
MAF	Melanoma- Associated Fibroblast
MAPK	Mitogen- Activated Protein Kinase
MDR	Multidrug Resistance
MFb	Myofibroblasts
MITF	Microphthalmia-Associated Transcription Factor
MM	Metastatic Melanoma
MTT	[3-(4, 5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide]
MTS	3-(4,5- dimethylthizol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4- sulfophenyl)-2H-tetrazolium
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NCCN	National Comprehensive Cancer Network
NIEHS	National Institute of Environmental Health Science
NFM	Nanofibers Mats
NMSC	Nonmelanocytic Skin Cancer
NO	Nitric Oxide
NOS	Nitrogen Species
NTU	Nanyang Technological University
NUS	National University of Singapore
OS	Overall Survivals
PBS	Phosphate- Buffered Saline
PCL	Polycaprolactone

PDGF	Platelet Derived Growth Factor
PEI	Polyethyleneimine
PET	Polyethylene Terephthalate
PFS	Progression- Free Survival
PSE	Proton- Sponge Effect
PK	Protein Kinase
PLGA	Poly (lactic-co-glycolic acid)
PLLA	Poly ($_L$- lactide)
PU	Polyurethane
PVP	Polyvinyl Pyrrolidone
RGP	Radial Growth Phase
ROS	Reactive Oxygen Species
RPM	Rotation per Minute
RT	Radiation Therapy
S	Solid
SC	Squamous Cell
SCCs	Squamous Cell Carcinoma
SE	Secondary Electrons
SEM	Scanning Electron Microscope
SG	Solid-Gas
SL	Solid-Liquid
SPF	Sun Photoprotection Factor
TAMs	Tumour Associated Macrophages
TCP	Tissue Culture Plate
TGFß	Transforming Growth Factor Beta 1
U.S	United States of America
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVR	Ultraviolet Radiation
VEGF	Vascular Endothelial Growth Factor
VGP	Vertical Growth Phase
WCA	Water Contact Angle

1 CHAPTER

INTRODUCTION

Nanotechnology is the science of materials at nano level. Nowadays, this technology is one of the fastest growing in the world, since is possible to create new material with advanced applications that may be used in many different fields.

Since the beginning of our civilization, man has had the need to establish dominance and the use of techniques in the manipulation of materials such as during the Stone Age, Bronze Age and further, the Iron Age (Zarbin, 2007).

The term nanotechnology was first used by Taniguchi in 1974, the publication of his work entitled "On the Basic Concept of Nanotechnology" is the combination of knowledge of materials at the Nano-scale or approximately 1-100 nanometers (Kennedy, et. al., 2003).

> *"Imagine a medical device that travels through the human body to seek out and destroy small clusters of cancerous cells before they can spread. Or a box no longer than a sugar cube that contains the entire contents of the Library Congress. Or materials much lighter than steel that possesses ten times as much strength".*
>
> U.S National Science Foundation

By using nanotechnology, it is possible to create things from very small raw materials, atoms and molecules which later, can be rearranged as desired propose. It is not a specific technology, but it is a set of techniques that are based on fundamentals of chemistry, biology, physics, materials engineering and computing, extending the human capacity to manipulate matter on an atomic level.

The technology of the 21st century is known as the "Nano World", with a greater scientific knowledge and greater understanding of the subject from the 90´s.

The word nanotechnology is used to define the various scientific and technical aspects that compromise Nanoscale materials, working and manipulating molecular structures and their respective atoms, or allowing the ability to build new materials and machines by the reorganization of atoms and molecules.

Nanomaterials are materials which have at least one dimension with a size less than 100nm (1nm= 0.000001mm), namely 80,000 times thinner than a human hair. A nanometer width is equivalent to three or four atoms, while nanotechnology refers to dimensions between 1 and 100 nanometers.

Currently, nanotechnology is being used in diverse areas ranging from medicine to the application and development of functional textiles. Here we can mention some companies that have large applications in nanotechnology are:

- Ciba Specialty Chemicals: has a line of products for encapsulating nanoparticles molecules on textile with antimicrobial purposes;
- Nano-Tex: technology developing nanoparticles to adhere to cotton or synthetic fibers;
- Nano- X: encapsulating nanoparticles for application in protective textiles;
- Schöller textiles: applications in nanoparticles for coating textile aiming to repel water and dirt.

The Nano-scale materials are of great importance regarding the development of functional textiles. It has great economic and commercial potential for the textile industry. By using nanotechnology, we can create and manipulate Nanofibers, Nanofilaments, Nanoparticles, Nano capsules, depending on their purpose. The use of this technology provides to the textile market, not only in Europe but also to the rest of the world, many possibilities to the product become more competitive, at low cost with increased profit margins. These are textile with high activity potential and better performance materials, which encourage these companies to invest constantly in research on new technologies, increasing competition and the development of this market, with the objective to meet specific needs of certain target group or application.

1.1 General Propose

This Ph.D. thesis proposes the conception and the development of new electrospun mat which has cytotoxicity (for melanoma skin cancer cells) and regenerative properties for human dermal fibroblast to achieve wound healing and skin regeneration by collagen release. The scaffold is thought to apply directly onto the injured area, which will ensure the delivery of therapeutic preparations with therapeutic effect

and dose into tumour cells and surrounded cells, whereby it may become an alternative treatment efficacy. This thesis involves two areas, namely, Human Medicine and Biomedical Materials Engineering.

The scaffold was produced by biodegradable polymers, approved by Food and Drugs Administration (FDA-the USA), the basis of which will be incorporated into composites electrospun mats, as an effective approach to treat melanoma cell *in vitro*, that will be an alternative approach in a near future to use for patient's, without requiring the use of invasive techniques, leading to a more efficient recovery and decrease the patient's malaise.

The composite electrospun mats are thought to work as drug delivery system (DDS), which can be made by the combination of different polymers. The anticancer activity for melanoma skin cancer will be tested, serving as a potential drug delivery vehicle for treating this specific disease. The electrospun mats can deliver efficiently the drugs little by little, promote good cell adhesion and good interaction of matrix to cells, and promote skin tissue regeneration, thus enabling accumulation of the drug in the target melanoma cells.

This Ph.D. thesis is of a great importance to the scientific field, since it is innovative and can serve as an alternative approach for patients which have been diagnosed with melanoma cancer. This might be in the near future a less invasive approach than the traditional therapies available on the market nowadays, such as chemo and radiotherapies.

1.2 Specific Goal

The specific goal of this Ph.D. thesis is to develop a scaffold with anticancer property to approach melanoma skin cancer in an early stage (*in vitro*). This study is thought to be useful and tested further *in vitro* and furthermore in human trials on the next few years. The scaffold should possess high cytotoxic properties of melanoma cells meanwhile promoting the growth and proliferation of healthy cells, as fibroblasts, aiming the skin regeneration. The skin regeneration can be achieved by expression of collagen by fibroblasts cells, once that collagen is the main structural protein in the extracellular space in several connective tissues in the human body, especially in the skin.

In resume, this thesis seeks:

- To explore the concepts of electrospun mats developed to the biomedical application;
- Investigate the anticancer properties of different composites mats;

- Develop an effective drug delivery system for melanoma skin cancer therapy;
- Investigate the regenerative properties and its possibility to apply as wound healing;
- Investigate the biocompatibility of the composites with healthy living cells;
- Prove the effectiveness of the investigated composite electrospun mats;
- Create new approach and scientific foundation for the development of the biomedical application of scaffolds, developed by electrospinning technique and its functionality.

1.3 Justification of the Work

Melanoma skin cancer is by far the most aggressive form of skin cancer. Especially in the United States, Australia, New Zeeland and Brazil, the incidence of this type of cancer continues to rise, although this is a worldwide issue. The American Cancer Society reported that it is estimated that 76,100 new cases of melanoma per year only in the USA and, approximately 9,710 people are expected to die due to the aggressive and metastatic form of melanoma.

On the past melanoma was known to develop mainly in older age group, particular men over the age of 65. In recent years, melanoma has been reported to be developed in young adults, especially women with age ranging from 25-39 years. This is pronounced increase in incidence rates, with bad prognosis if is not diagnosed in early stages, often with severe outcomes.

Sun exposure is the major risk factor for melanoma cancer, especially in tropical countries, although the incidence of melanoma can also be related to others factors that influence incidence rates, such as immunosuppression, genetic susceptibility, family history of melanoma and environmental factors.

Chemotherapy treatment was the mainstay approach option for the advanced- stage melanoma, prior to the recent therapeutic advances. Several studies have investigated various chemotherapy combinations, as well radiotherapy in other to expand the clinical responses, but these appears to be not effective in overall survival rate. In addition to that, most of these therapies can worsen the patient health condition, due to invasive approach. Chemo and radiotherapies can promote apoptosis of malignancy cells, but at the same time have the apoptotic effect in healthy surrounding cells.

In this way, it appears pertinent and current the need to mediate a new version for the treatment of melanoma, in relation to the existing methods available on the market. In addition, it is pertinent to evaluate the release and efficiency of the controlled drugs through scaffolds, namely electrospun mats,

using biodegradable and biocompatible polymers for its formulation and development. Therefore, reducing the use of invasive therapies such as chemo and radiotherapies.

In this context, the main objective of this work was defined as the development of a new alternative approach for melanoma skin cancer, which can provide apoptosis of malignancy cells, accelerate the wound healing process and promote skin regeneration through the interaction of cells to cells and cell to the matrix, focusing the growth and proliferation of healthy cells, providing good adhesion of cells to the scaffold surface and nutrients supply necessary for human dermal fibroblast cells proliferation, and collagen expression.

1.4 Applied Methodology

Firstly, a theoretical study through literature review was conducted in order to comprehend and define the problems, regarding the Melanoma Skin Cancer treatment.

In a second stage an exploratory research to characterize the problem and suggest solutions via *in vitro* studies, that may be useful in the near future as a less invasive approach with a minimized side effect to patients undergoing cancer treatment, namely melanoma.

The third stage included the development of copolymer nanofibrous scaffolds in different concentrations. Therefore, we conducted characterization and influence of certain parameters as:

- Process parameters variables: Electrical potential, flow rate, the concentration of the solution and distance between the Taylor Cone and the collector;
- Environmental parameters which affect the process: velocity of the air in the chamber, moisture, and temperature;
- Operating parameters: rate flow, electric field strength and the electric current.

Quantitative analysis, FT-IR, water contact angle, mechanical properties and morphological characterization of the scaffold by SEM.

In vitro studies were performed to provide reliable data of toxicity for melanoma cells B16, and biocompatibility cells of human dermal fibroblasts for all electrospun scaffolds developed. The collagen release profile was also investigated. We made all the practical and experimental work to produce and test electrospun scaffolds with all the desired properties.

1.5 Outline

This thesis is divided into five chapters, starting from the literature review to the practice and realization of this research work. The chapters developed are here particularized as follow:

- **Chapter 1.** Refers to the framing of the thesis. In this sense, the specified problematic synthesis of the existing information, specific goals for this proposed research, the justification of the work, the methodology used, such as the structure of the thesis.
- **Chapter 2.** We conducted a literature review and the state of the art regarding melanoma skin cancer. This chapter is crucial to understand the importance of the contribution for the scientific community. For this reason, we divided this chapter into four main topics. Firstly, we present an overview of skin morphology, including some types of skin cancer such as Basal Cell Carcinoma (BCC), Squamous Cell Carcinoma (SCC) and Melanoma which is the main focus of this doctoral thesis. In this context, it was important to elucidate the melanoma microenvironment, the association of melanoma cancer development associated to human dermal fibroblasts, melanoma initiation, and progression through hypoxia-inducible factors (HIF), TAMs, and some conventional therapies mostly used to treat cancer as chemo and radiotherapy. In the second part of this chapter we introduce the nanofibers composites. We briefly explain the fabrication methods, the nanofibers properties, and an overview of the FDA approved composites used to conduct the practical part of this thesis. We detail the polycaprolactone (PCL) and Linear Polyethylenimine (LPEI) and Branched Polyethylenimine (BPEI). The third part is dedicated to the biomedical application of nanofibers composites. Here we elucidate some important characteristics of drugs delivery system, the permeation drug´s pathway, the implication of electrospun based materials for DDS in healthcare, used in wound healing process and cancer therapy. Last but not least, we present the biofunctionalization of fibrous electrospun scaffolds. We also report the characterization of cells to nanofiber, some cytocompatibility assays as MTT and MTS, and the cells used in this research work.
- **Chapter 3.** We clarify the experimental part of the thesis and different assays that we used to develop the practical part. We present the development of the electrospun mats, the characterization of the scaffolds developed such as water contact angle (WCA), morphological characterization of the electrospun mats by SEM imaging, FT-IR, and determination of mechanical properties. This is followed by the description of cell culture methods, including human dermal

fibroblasts and murine melanoma cells (B16). It is also reported the biocompatibility assays used as CMFDA, Sirius Red Collagen, and MTS.

- **Chapter 4.** We conduct detailed interpretation of all outcome data obtained from the above-mentioned experiments.
- **Chapter 5** presents the final considerations, an overview results for this thesis and future research guidelines.

The present study starts with a perspective of developing a more efficient biomaterial for the treatment of skin cancer, as a more efficient alternative than the traditional approaches as radio and chemotherapy, aiming to avoid side effect and at the same time promote the regeneration of tissue, acting as support for extracellular matrix (ECM).

1.6 Original Contributions

During these years, part of this study has been presented and recognized by the scientific community, through the following listed original contributions in international journals, conferences, and books:

Articles in Scientific International Journals

- **Naves, L. B.**, Almeida, L., Ramakrishna, S. (2017). Understanding the microenvironment of Melanoma cells for the development of target drugs delivery systems. *European Medical Journal- Oncology*, *5*(1), pp. 85–92.
- **Naves, L. B.**; Dhand, C.; Almeida, L.; Rajamani, L., Ramakrishna, S. (2017). Linear and Branched Polyethynimines for skin regeneration treatment *In Vitro* Study. *J Pharm Drug Deliv Res*, *6*(4), 73. Available in: http://doi.org/10.4172/2325-9604-C1-020
- **Naves, L. B.**, Almeida, L., Marques, M. J., Soares, G., & Ramakrishna, S. (2017). Emulsions Stabilization for Topical Application. *Biomaterials and Medical Applications*, (65), pp. 1–7.
- **Naves, L. B.**, Dhand, C., Venugopal, J. R., Rajamani, L., Ramakrishna, S., & Almeida, L. (2017). Nanotechnology for the treatment of melanoma skin cancer. *Progress in Biomaterials*, *6*(1–2), pp. 13–26. Available in: http://doi.org/10.1007/s40204-017-0064-z

- **Naves, L. B.**, Dhand, C., Almeida, L., Rajamani, L., Ramakrishna, S., & Soares, G. (2017). Poly(lactic-co-glycolic) acid drug delivery systems through transdermal pathway: an overview. *Progress in Biomaterials*, *6*(1–2), pp. 1–11. Available in: http://doi.org/10.1007/s40204-017-0063-0
- **Naves, L. B.**, Dhand, C., Almeida, L., Rajamani, L., & Ramakrishna, S. (2016). In vitro skin models and tissue engineering protocols for skin graft applications. *Essays in Biochemistry*, *60*(4), 357 LP-369. Available in: http://doi.org/10.1042/EBC20160043
- **Naves, L. B.**, & Almeida, L. (2015). Wound dressing for melanoma skin cancer therapy. *Autex Research Journal*. Available in: http://doi.org/978-606-685-275-3

Articles in the Proceedings of International Conferences

- **Naves, L. B.**, Dhand, C., Almeida, L., Rajamani, L., Venugopal, J., Ramakrishna, S. (2017). Polycaprolactone blended with Linear and Branched Polyethyleneimines scaffolds for skin regeneration treatment- In vitro study. In Proceedings of *International Conference and Exhibition on Nanomedicine and Drug Delivery*, Osaka (JP). Available in: https://www.omicsonline.org/abstract/polycaprolactone-blended-with-linear-and-branched-polyethynimines-scaffolds-for-skin-regeneration-treatment-in-vitro-study/
- **Naves, L. B.**, & Almeida L. (2015). Wound healing dressing and some composites such as zeolite, TiO_2, chitosan and PLGA: A review. In Proceedings of the World Academy of Science, Engineering and Technology, Miami (USA). Available in https://waset.org/author/l-b-naves
- **Naves, L.** B., & Almeida, L. (2015). New Approach for melanoma skin cancer controlled releasing drugs for neutron capture therapy: A review. In Proceedings of The Fiber Society 2015 Fall Meeting and Technical Conference, Raleigh (USA). Available in http://thefibersociety.org/Portals/0/Past%20Conferences/2015_Fall_Abstracts
- **Naves, L. B.**, Roshan, P., & Belino, N. (2014). Functional design as a tool for children undergoing chemotherapy treatment. In Proceedings of the *Global Fashion Conference* 2014, Ghent (BE). Available in: http://doi.org/978-989-20-5337-0

Books

Naves, L. B., & Almeida, L. (2015). *Approaching Skin Cancer Through Textile Engineering Perspective - A Review* (1st ed.). Saarbrücken: LAP LAMBERT Academic Publishing.

Chapter in Books

- **Naves, L. B.**, Almeida, L., & Rajamani, L. (2017). Nanofiber composites in skin tissue engineering. In *Nanofiber Composites for Biomedical Applications* (1st ed., pp. 275–300). Chennai - India: Elsevier Woodhead Publishing. Available in: http://doi.org/10.1016/B978-0-08-100173-8.00011-9

2 CHAPTER

LITERATURE REVIEW AND STATE OF THE ART

2.1 Skin Cancer Problem

2.1.1 Contextualization of the Problem

One of the most aggressive types of skin cancer is Melanoma. The melanoma cancer is originated from the malignant transformation of melanocytes. It has low survival rate, easy to relapse and a notorious high multidrug resistance (MDR). In 2014, only in the United States, it has been reported that nearly 76,100 people were diagnosed with new cases of melanoma, and estimation of 9,710 was expected to die. The trend worldwide is to use more and more drugs and nanotechnologies, for biomedical applications (Naves et al., 2017a).

Nanotechnology is the field of applied science focused on the design, development, fabrication, characterization, and application of devices and materials in nanoscale. It has been shown a potentially significant impact of nanotechnology on healthcare by delivering changes in drugs delivery, disease monitoring, and diagnosis, regenerative medicine, implants, as well as research tools for biomedical science and drugs delivery. The use of nanotechnology in the healthcare environment can create new alternative treatments, minimizing the side effects, more efficient due to the target compounds, consequently minimizing the drug dosage and the treatment cost either to the patient or to the government. In addition to that, nanotechnology has been applied to most conventional melanoma therapies.

In the last few years the researchers have been focusing their research on discovering new alternative approaches to treat cancer using nanotechnology. This approach has demonstrated that nano delivery of drugs for chemotherapy, immunotherapy, target therapy and photodynamic therapy has increased the treatment efficacy. In 2017, Naves and his co-workers stated:

> *"Targeting in nanotechnology refers to the spatial localization of the nanoparticles within the intentional sites and is distinct from molecularly targeted drugs. Targeted drug means blocking essential biochemical pathways or mutant proteins that are required for tumour cell growth".*
>
> (Naves et al., 2017a)

Nanofibers have been incredibly used for the biomedical and sustained drugs delivery systems (DDS). Nanofibers are ideal for this approach due to their characteristics, their dimensions are similar to the components of the native extracellular matrix, possibility to mimic the fibrillar structure, which can provide essential cues for cellular survival and organization functions.

Based on melanoma skin cancer issue, we propose to develop a new alternative approach for the treatment of this type of cancer, which will be developed by using electrospinning technique and ascertain that the scaffolds have some specific characteristics for biomedical application, such as cytotoxicity to malignancy cells, biocompatibility with healthy cells, acting as tissue regeneration, working as an extracellular matrix, providing great cells adhesion, growth and proliferation rate with the necessary nutrients supply. The main goal of this Ph.D. thesis is the development of a less invasive and more effective approach for the treatment of melanoma skin cancer. The essays in this thesis will be carried out *in vitro* only.

2.1.2 Skin Morphology

Skin is the largest organ in the human body, in adults, it represents 8% of their total body mass and has the surface area of about 1.8 m^2. The skin is mainly composed of three different layers, namely the epidermis, dermis, and hypodermis as we can observe in **Figure 2-1**, these layers are mainly composed of nerves and blood supply. All these layers are responsible for protection from the risk that might be posed by thermoregulation and surrounding environment (Naves, 2013). The main two layers are the epidermis and dermis. The epidermis is outer layer, surrounded by extracellular lipid matrix, corneocytes and keratinocytes (which produce keratin), both packed into the extracellular lipid matrix (arranged in bilayers) some studies reported this arrangement as "brick and mortar", separated from the dermis by basement membrane, and melanocytes (which provide pigmentation for the skin). The epidermis is responsible for promoting a protective barrier against bacteria or even harmful toxins. The dermis is located just under the epidermis, formed by a variety of connective tissues, as nerves, lymphatic system,

many types of cells and blood vessels (Elias & Menon, 1991). The dermis is formed by extracellular matrix (ECM), formed by glycosaminoglycans (GAGs), collagen and elastin. The functions are foremost as a barrier, working as a regulator for water retention and heat loss, sensory organ and preventing pathogens from entering the body. The primary type of cell found in the dermal layer is the fibroblast; these cells are responsible for synthesizing ECM proteins and enzymes, and activate the response to wound healing process by protease and collagenases. The innermost layer is called hypodermis, which is responsible for thermoregulation and mechanical properties of the skin (Boughton & Mclennan, 2013). In addition to that, skin is an attractive model to test novel drugs and regenerative medicine, with emphasis on skin tissue regeneration for chronic or acute wounds. The skin surface is a potential route for local and systemic drugs delivery, using either nanoparticles or electrospun mats. The delivery of the drugs can also be reached into opened follicles of the hair. One of the primary and most important functions of skin in mammalian is to provide a protective barrier against UV radiation, fungi, bacteria, and any nanoparticle that might come from the external and natural environment (Naves et. al., 2017a).

Naves and Almeida (Naves & Almeida, 2015) stated:

> *"In human's skin, the hair follicles cover only 0.1% of the skin surface, however, target drug delivery can be performed through follicular route, to achieve a better drug penetration. By pulling out the hair sheath, it is possible to make a significant penetration of medicines in a size smaller than 40 nm into the epidermal cell, to reach, and facilitate a higher topical nanoparticle delivery through the skin. Mechanical stress on the skin is necessary, which can be achieved by massage, skin flexing, or follicles pulled out. Over the last few years, the interest in a target by follicular delivery has increased as it might be a potential delivery route into sebum to control the skin diseases."*

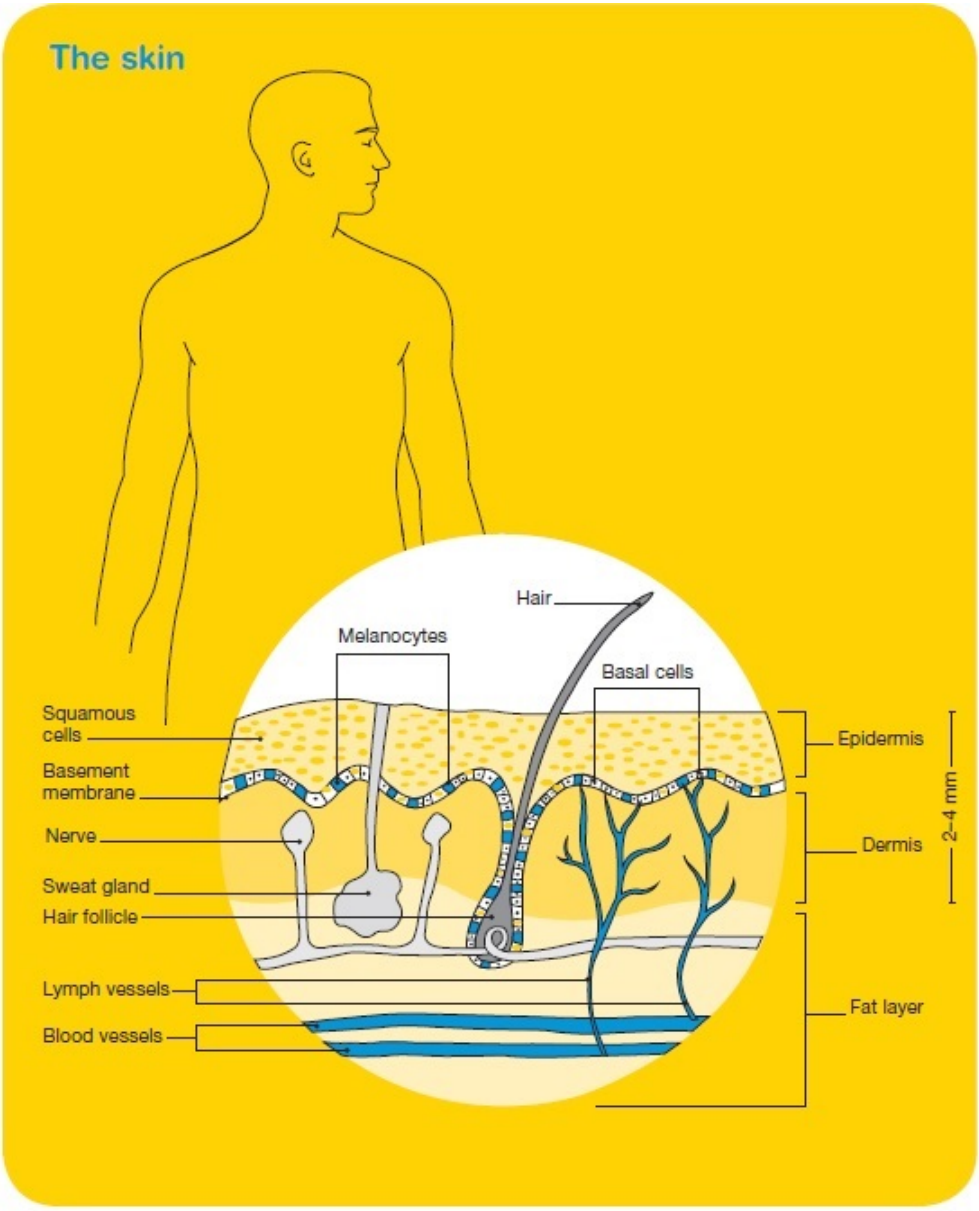

Figure 2-1 Schematic representation of the skin layers, namely the epidermis, dermis, and hypodermis. Source: (Grove, 2016) p.7.

Topical drugs delivery systems have been investigated and practiced since the end of the 70´s, by using either nanoparticles or transdermal patches to be administrated as readily available skin localized therapy (Roy et. al., 1996).

The pH of the skin surface is directly influenced by several factors such anatomical site, sweat, gender and hydration (Tinkle et al., 2003). Usually, the natural skin pH is around 4.2 to 5.6 (Schmid-Wendtner & Korting, 2006). The pH of the skin surface is very important for degradation of drugs when approaching it through the transdermal pathway, it also supports nanoparticles penetrations. Electrostatic force can be decreased by a solution with lower pH (Murphy et. al., 2010).

A natural process that occurs in the human skin is the desquamation, which happens in the Squamous Cells (SC) layers. The whole process of renewing the skin surface is completed in approximately 14 days. This process is very important for our protection, once that the corneocytes can provide elimination of certain matters such as pathogens, solid particles or even cancer cell. It is important to keep in mind, that the desquamation process may vary from one person to the other accordingly to the anatomy, malaise, and age (Naves et al., 2017a; Reddy, Guy, & Bunge, 2000).

2.1.3 Types of Skin Cancer

Our body is constantly making new cells, aiming growth, proliferation, replace dead cells, heal injuries and replace worn-out tissue. The cells, normally multiply and die in an orderly way. The cancer is formed by cells disease, when cells divide and proliferate in an abnormal way, which may lead to lymph or blood fluid in the body, thus, forming a lump called tumour.

A tumour can be either benign or malignant. In the first type, the cells are not considered as cancer, once that the cells are confined to one specific area and they are not able to spread to other body sites. A malignant tumour takes place when is made up of carcinogenic cells, having the ability to spread through the body via the lymphatic system, also known as lymph fluid, and via the bloodstream. It is possible to observe at the upper part, how the cancer starts, by the proliferation of abnormal cells, when the angiogenesis takes place, cancer becomes invasive or malignant.

The primary cancer is the type of cancer that first develops in a tissue or organ. A malignant tumour is named when a malignant tumour affects some type of cells or the organ. Differently, localized cancer is a malignant tumour that has not spread to the other body site. As mentioned above, in the angiogenesis process, the tumour may invade deeper the surrounding tissue, by vertical growth, and grow its own blood vessels.

In **Figure 2-2,** it is also possible to observe how cancer spreads, also known as the metastatic effect. The metastasis or secondary cancer happens when the carcinogenic cells grow and form another tumour at a new body site. Always when there is a metastatic effect, it remains the name of original cancer. As example, we can mention skin cancer, when it spread to the lymph nodes is called metastatic skin cancer. Basal cell carcinoma rarely spreads, while squamous cell carcinoma and melanoma may easily lead to metastatic effect (Grove, 2016).

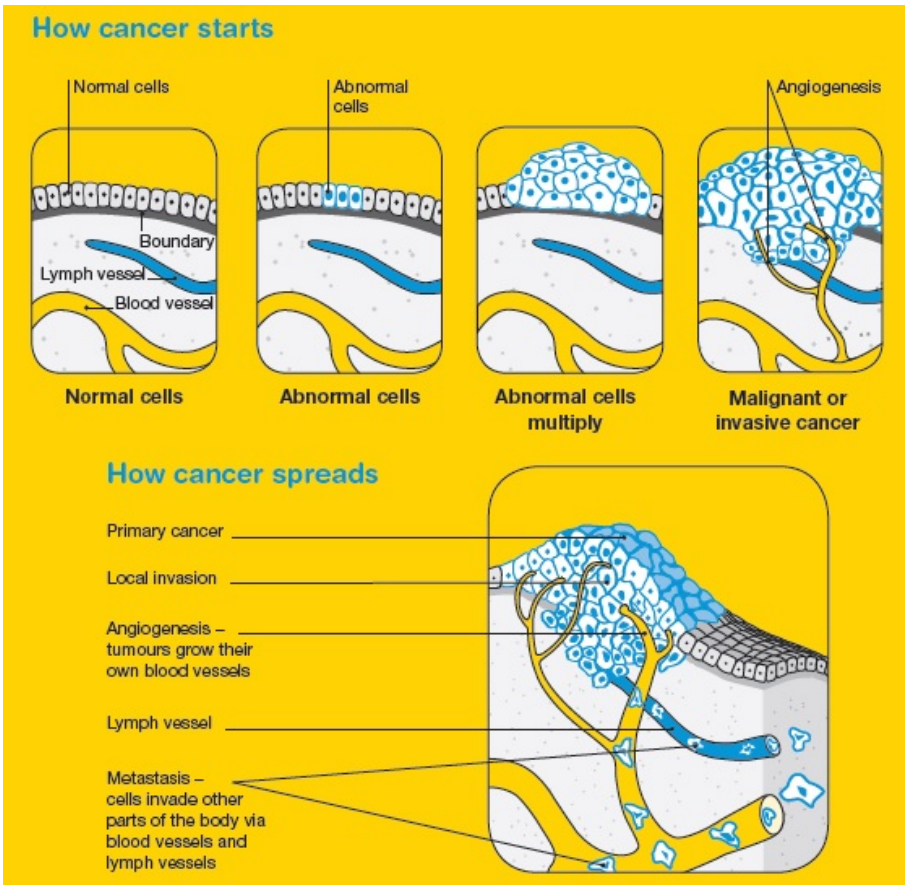

Figure 2-2 Schematic carcinogenic cells growth and proliferation, upper image. Representation of metastatic effect lower part. Source: (Grove, 2016) Adapted.

It is well known that skin cancer is by far the most malignancy of humans, especially in the white population. It was reported that over a million cases are detected each year (D'Orazio et al., 2010). The skin cancer is named according to the clinical behavior and the cells which they arise from. The three commonest types will be better explained in this topic, we will give a greater focus on Melanoma, due to the purpose of this study.

Briefly, the types of skin cancer are referred as nonmelanocytic skin cancer (NMSC) are: basal cell carcinoma (BCCs), and squamous cell carcinoma (SCCs). Cutaneous malignant melanomas (CMs), appears in the literature also reported as melanoma or malignant melanoma (Narayanan, Saladi, & Fox, 2010).

Skin cancer as many other types of cancers have increased the incidence significantly with age, environmental etiologies (UV exposure), and, reflecting long latency between cancer establishment and carcinogen exposure. Many cases of skin cancer remain not reported and the incidence of these type of

cancer continues increasing dramatically. In **Table 2-1,** it is shown a substantial underestimation of the epidemiological data.

Reports from Europe and the United States, suggest that the incidence of NMSC is progressively increasing. Each year, worldwide, are expected 2-3 million cases of NMCs. The incidence may vary, although there is a high incidence in Caucasians population (Simões, Sousa, & Pais, 2015). The overall observed in most parts of Australia, Canada, Europe, and U.S.A, show that the incidence of skin cancer is increasing from 3% to 8% per year. Over the next 30 years, the incidence rate is expected to double (Rhee et al., 2007). In Europe, it is reported that NMSC mortality rate is lower in Nordic countries and higher in women and men in southern European countries such as Italy, Portugal, Greece and Spain (Boyle et. al., 2004).

Table 2-1 Overview of epidemiological data of BCC, SCC, and CM. Source: (Simões et al., 2015) Adapted.

Highlights of epidermal data		
Basal Cell Carcinomas (BCCs)	Squamous Cell Carcinomas (SCCs)	Cutaneous Malignant Melanoma (CMs)
2-3 million cases of NMSCs occur worldwide each year (Narayanan, D.L., Saladi, R.N., Fox, 2010). Only in the U.S 1,3 million cases each year (Rhee et al., 2007) Rates are expected to double in the next 30 years (Rhee et al., 2007). NMSC mortality rate is lower in Nordic countries and higher in women and men in southern European countries such as Italy, Portugal, Greece, and Spain (Boyle et al., 2004).		65 thousand people die per year worldwide (Narayanan, D.L., Saladi, R.N., Fox, 2010).
highest rates in elderly men and increasing incidence in young women (O'Driscoll et al., 2006).	Increasing rates, although it varies depending on the country (Lomas, Leonardi-Bee, & Bath-Hextall, 2012).	Incidence rates are at least 16 times greater in Caucasians than African Americans and 10 times greater than Hispanics (Battie, Gohara, Vershoore, & Roberts, 2013).
In the US, 30% of all new cancers diagnosed are BCC (Rittié et al., 2007).	SCC has higher possibility to invade other tissue and cause more death than BCC (Suárez et al., 2007).	132 new cases worldwide will occur each year (Narayanan et al., 2010).

In **Figure 2-3** are presented images of Basal cell carcinoma and squamous cell carcinoma, both keratinocyte skin cancer, and Melanoma superficial and modular. It is possible to observe the difference regarding the color, the nodule and the shape of each type of cancer.

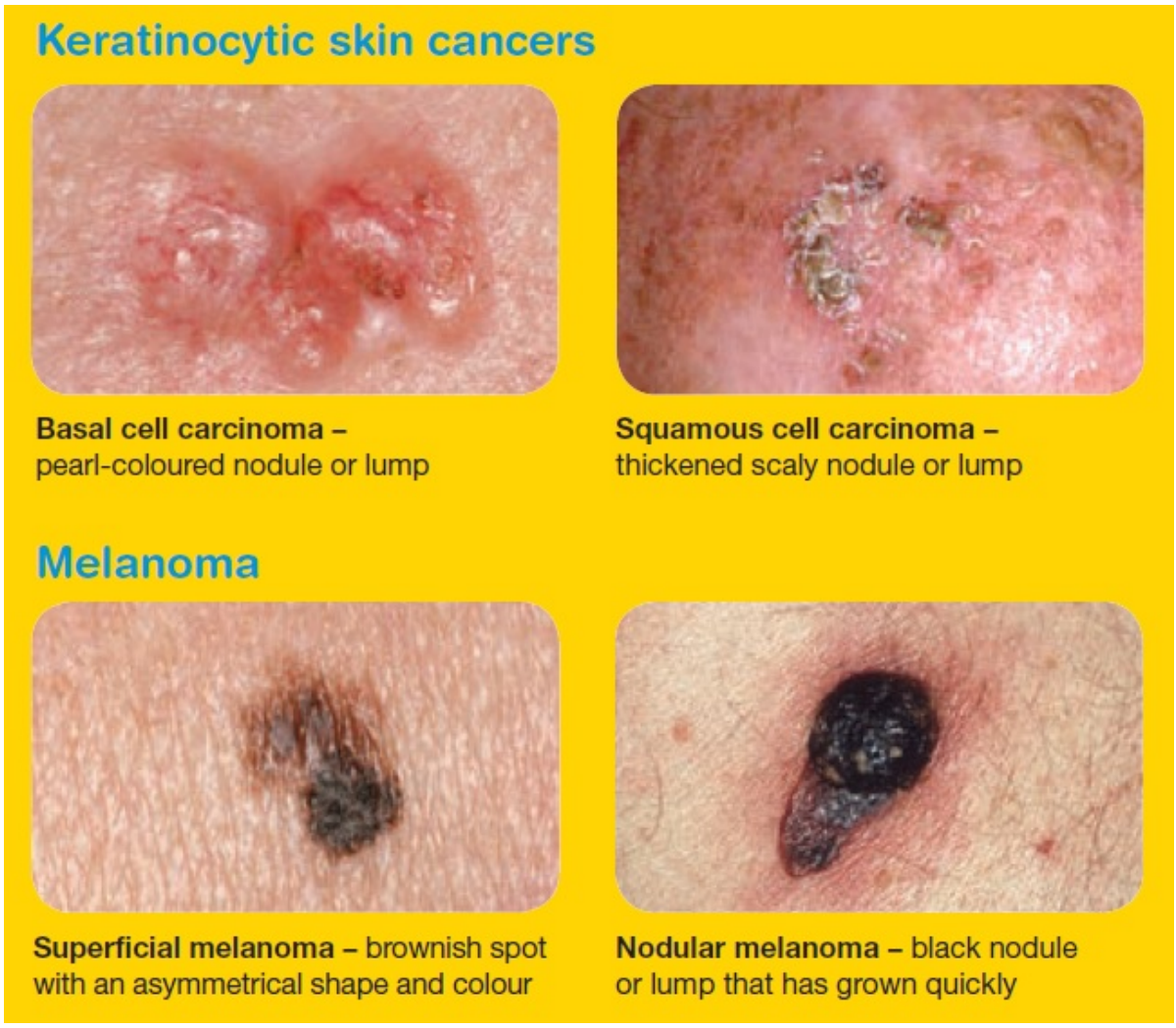

Figure 2-3 Images of skin cancer, namely BCCs, SCCs and melanoma superficial and nodular. Source: (Grove, 2016) p. 13, Adapted.

2.1.3.1 Basal Cell Carcinoma (BCC)

Basal cell carcinoma is related to 80-85% of all NMSCs. This type of cancer is the most common malignancy in the Caucasian population. In addition to that, it has a very low rate of mortality and metastatic effect to other body sites (Lang & Maize, 2005). Naves & Almeida stated (Naves & Almeida, 2015):

> "*... the incidence of UV rays are directed related to high risk of incidence of BCC skin cancer. However, the incidence of BCC is inversely related to inherent melanin content in the skin, serving as a protective function. It is uncommon for darker skin to get BCC."*

Controversially, if the population with darker skin suffers from albinism, which is when the melanocytes fail in the production of melanin, the risk of BCC incidence is substantially increased (Alexander & Henschke, 1981).

Some physicians recommend exposure to ultraviolet light therapy for the treatment of some inflammatory skin conditions such as psoriasis, which has been also reported in the literature, that this type of radiation may cause an increase the BCC risk (Forman et. al., 1989).

The treatment for BCCs mostly of the time is indicated with curettage and electrodesiccation (CE). However, when the patient has a recurrent tumour, tumour size larger than 2 cm, morpheaform tumours or in tumour in thickly hair-bearing areas, the electrodesiccation is not recommended due to its lower rate of success (Werlinger, Upton, & Moore, 2002).

2.1.3.2 Squamous Cell Carcinoma (SCC)

Reported as the second most common type of skin cancer, Squamous cell carcinoma, occurs particularly in the sun-exposed areas, such as neck and head. It has been reported that the majority of incidence of Squamous cell carcinoma, is caused and related to the mutation at the gene p53, which is the tumour suppressor. This mutation takes place by the ultraviolet light exposure (Sarasin & Giglia-Mari, 2002). Differently, from Basal cell carcinoma, the squamous cell carcinoma has a metastatic effect.

Tward and co-workers, reported in their study (Tward et. at., 2012):

> *"SCC begins with the squamous cells in the epidermis which are situated directly above the basement membrane where the basal cells are located in the stratum basale (...) as the keratinocytes, the squamous cells undergo an abrupt transition in the stratum granulosun of the epidermis where the intracellular keratin filaments clump, the cell undergoes programmed death, and a keratogenesis shell remains with intracellular was esters acting like "mortar" between keratinocyte "bricks" forming the water light barrier of the outer layer of the skin, the stratum corneum. Because of the abundance of keratin protein in squamous cells, in general, SCC is clinically much scalier and crusted than BCC."*

It has been also reported that, due to the decrease of immunosurveillance, patients that underwent solid organ transplant and with lymphoproliferative malignancies become more prompt to develop SCC. As an example, we can mention patients who have done a renal transplant. The recipient of the organ is more

vulnerable, he has 7% of chance to develop SCC in the first year after the transplant. This rate increases to 45% after eleven years, and unfortunately, drastically, to 70% risk of incidence after 20 years of transplantation. When considering patients heart transplantation, this situation becomes worse, 2-3 times greater risk of SCC incidence compared to those, whose received renal transplant (Bouwes Bavinck et al., 1996).

Both types of skin cancer BCC and SCC are related to the amount of ultraviolet radiation exposure received, and inversely proportional to the degree of skin pigmentation in the population, meaning that the darker the skin the lower is the incidence of NMSCs. Although many patients with NMSCs may remain undiagnosed and unregistered, the rate is increasing 10% worldwide, especially, in young women and older men individuals. The reported NMSCs are often found on sun-exposed areas such as head and neck (Diepgen & Mahler, 2002).

2.1.3.3 Melanoma

Melanoma skin cancer is one of the most common types of cancer. It is known that in women is the seventh leading cancer and in men is the fifth in the United States. Annually, 800,000 people are expected to develop melanoma skin cancer. Over the last few years, the incidence of Melanoma skin cancer has increased substantially, especially in women. It is most common among Caucasians with green or blue eyes and blondish (Scotto, Fears, & Fraumeni, 1982). Approximately one in 100 newborns will at some stage of their lives develop malignancy in their lifetime (Kopf et. al., 1995).

This type of cancer is the most aggressive skin cancer and represents only 3% of all diagnosed skin cancer in the United States. The high rate of patient's death is related to advanced melanoma metastasis, which usually occurs several months up to years after the primary melanoma diagnosis. At an early stage, the melanoma tumour can be removed, therefore promoting a survival rate up to 99% (Naves et al., 2017a). When the patients are diagnosed with the late stage of melanoma, cancer may have a metastatic effect, thus, spreading the malignancy cells to the other parts of the body.

When the rate of melanoma skin cancer is compared in terms of different races, the rate in Caucasians is 10 times greater than in Hispanics and 16 times greater than in African American. It has been reported that people with darker skin have higher mobility and fatality, when compared to other skin tons, once that they are undiagnosed for a while. In African American, the incidence rate is lower, because this group has increased epidermal melanin, resulting in a Sun Photoprotection Factor (SPF) of up to 13.4 in African

American skin. Another study suggested that the epidermal melanin can filter twice as much as UVB radiation in African Americans as does in Caucasians.

In the literature review, it has been reported the incidence and correlation of UV radiation and melanoma rates. The UV radiation of sun exposure may cause irreversible damage; both UVA and UVB can damage directly and indirectly the DNA. Once these mutations occur in the DNA, they lead to the modification of the DNA, which will further develop skin cancer (Soehnge, Ouhtit & Ananthaswany, 1997). There are 5 important factors that might affect the incidence of UVR levels reaching the earth's surface such as:

1. Clouds and others: at higher cloud-cover densities there are lower UV levels. On the one hand pollutants, haze, and fog can decrease UV incidence by 10-90%, and on the other hand sand, metals, and snow can reflect it up to 90% (W. H. Organization, n.d.);
2. Shades: the shadows may protect the UVR exposure for up to 90% according to different types of shade, e.g., dense foliage had the highest protection, while an umbrella at the beach shows low levels of solar radiation protection;
3. Seawater: can reflect UV rays up to 15%;
4. Latitude: living closer to equator zones increases the incidence of solar radiation, thus maximizing the probability of skin cancer incidence. The lower the latitude, the higher is the UVR incidence, on the other words, the UVR must travel a shorter distance through ozone-rich portions of the atmosphere and in turn, more UVR is emitted (Lautenschlanger,Wulf & Pittelknow, 2007);
5. Altitude: every 1000m increasing in elevation, the UVR intensity increases by 10-12% (W. H. Organization, n.d.).

In addition to the external process that may lead to skin cancer, the use of artificial UV tanning can also be linked to skin cancer development. Only in the US, approximately 28 million Americans are reported to use the artificial UV tanning. The exposure to the tanning bed and sunlamps have been warned to be carcinogenic by the National Institute of Environmental Health Science (NIEHS). The effects of UV exposure either natural or artificial may take 20 years to result in skin cancer (Chen et. al., 1998; Spencer & Amonette, 1995). In **Figure 2-4** is possible to observe the UV index and alert in Australia, published by the Cancer Council of Australia (Grove, 2016).

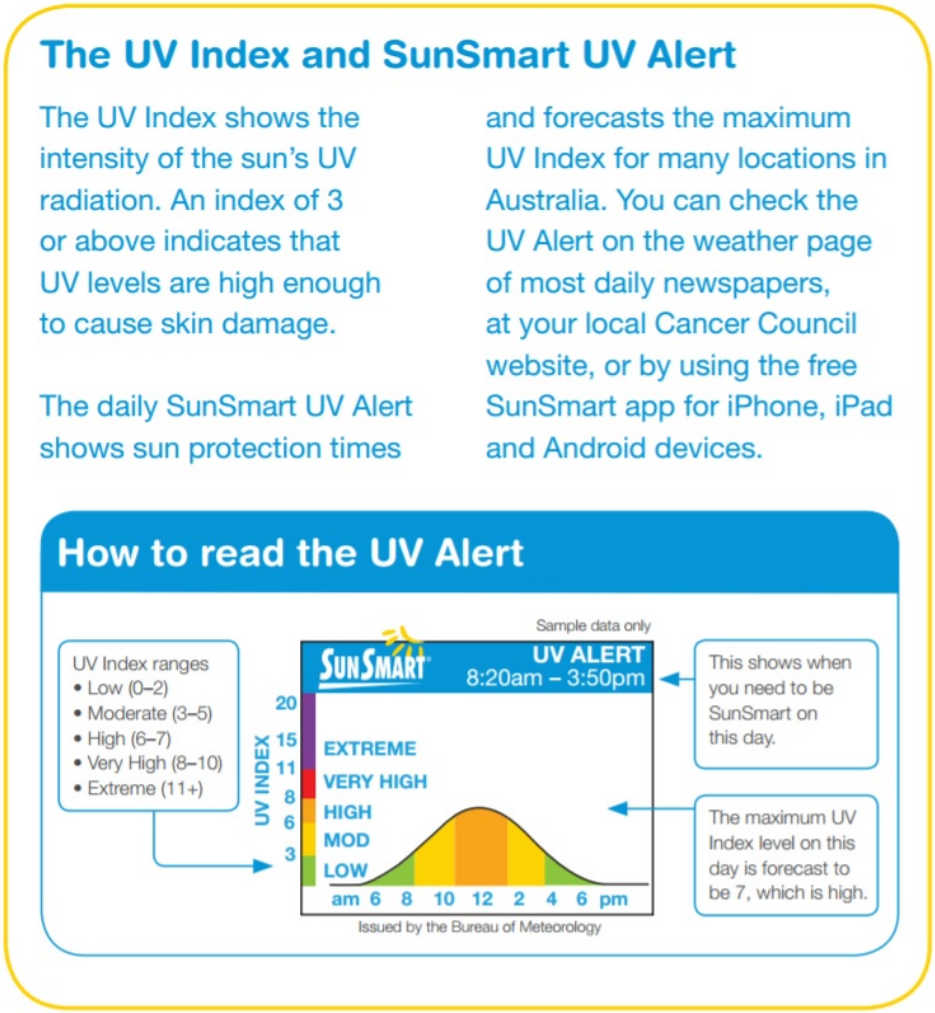

Figure 2-4 UV index and alert in Australia. Source: (Grove, 2016) p. 16.

Naves and co-workers, stated (Naves, Almeida, & Rajamani, 2017b):

> *"In skin carcinogenesis, UV immunosuppression is considered an important event. The exposure to UV affects the skin immune system as delayed-type hypersensitive reactions, presenting cell function and inducing immunosuppressive cytokine production. Many cellular functions are provided by the p53 tumour suppressor gene, among all these functions we can distinguish transcription, cell cycle differentiation, regulation, and inhibition. The gene p53 is also responsible for DNA repair. Therefore, if the gene p53 is mutated, it will no longer aid in DNA repair process, as the result of this mutation, there is deregulation in the cellular cycle, and a proliferation of mutated keratinocytes, initiating skin cancer".*

Fortunately, this paradigm has changed due to the advances done in new pathways and targeting in drugs delivery systems (DDS), which may play a major impact on the development of immunotherapy and target therapy for melanoma cancer. The most common types of drugs used for the treatment of melanoma metastatic effect are ipilimumab and vemurafenib, both approved by the U.S. Food and Drug Administration (FDA), although both therapies still present their limitations. These drugs play different role targeting in melanoma cells, Ipilimumab can achieve durable benefits to the target cells by blocking the immune suppression of T cells which is induced by cytotoxic T lymphocyte antigen 4 (CTLA-4), however, around 80 to 85% of the patients do not respond to this therapy. Vemurafenib can achieve rapid tumour regression, targeting melanoma harboring BRAFV600E mutations. The negative side of this drug is that patients might have drug resistance after six months of treatment. In 2016, Wang and Yun, presented the melanoma network environment affecting the development of melanoma focusing the tissue hypoxia, macrophages and stromal fibroblasts (Wang & Yun, 2016). It is only possible to develop new drugs and strategies for melanoma therapy, prognosis, and diagnosis through a better understanding of how tumour microenvironment can directly affect the melanoma cancer progression.

In a recent study published by Wang and colleagues (Wang et al., 2015), they reported the anticancer activity on melanoma cells of curcumin (CUR) loaded electrospun nanofibers with enhanced bioavailability. CUR, has many advantages, as anticancer activity and antioxidant (Bar-Sela, Epelbaum, & Schaffer, 2010; Maheshwari et. al., 2006; Shishodia, Chaturvedi, & Aggarwal, 2007). In this study, the authors used a water-soluble polymer polyvinylpyrrolidone (PVP) for the development of dissolution CUR, aiming the enhancement of the bioavailability.

For the preparation of electrospinning solution, the researchers dissolved 1 g of PVP in 10 mL of acetic ether, then they loaded different concentration of CUR, related to the concentration of PVP in the solution. The CUR concentrations used in the study were 10%, 15%, and 20%. Using flow rate of 2 mL/h, the distance from the needle to the collector was 15 cm, and the voltage applied of 15 kV.

They cultured murine melanoma cells line, known as B16. For the studies *in vivo,* they tested 48 male mice C57BL/6. For anticancer activity assay, the free CUR and PVP blended with CUR electrospun mats were administrated orally at a dosage of 25 mg/kg. The injection of B16 cells was inoculated and administrated as subcutaneous allografts, at a concentration of 1 x 10^6 B16 cells in PBS. The humanized euthanasia was carried to all mice after the study by pentobarbital sodium overdose injection. The tumour of each mouse was further dissected and kept in 4% formalin and PBS solution for fixation, of a tumour.

Under the excitation of 447nm CUR electrospun mats were observed giving the fluorescent red color **Figure 2-5 (A), (B)** and **(C)**, the PVP nanofibers did not show any fluorescence, because it does not have this behavior. The FTIR spectra data is shown in **Figure 2-5 (D)** and **(E)**. They reported that there was no sign of chemical changes as a result of electrospinning approach, either for CUR or PVP, retained their individual characteristics. In the FTIR analysis, significant peaks were attributed to intermolecular hydrogen bonding. Significant peaks can be observed, the peaks at 1602 and 1508 cm^{-1} are related to the aromatic stretching of C = C, CUR blended with PVP show 3600 – 3400 cm^{-1}.

In **Figure 2-6**, it is shown the anticancer histology images of a tumour induced by B16 cells. It is possible to make a comparison of three different tumour size, from the left to the right, we can observe the control, the tumour size of the mice treated with CUR electrospun, and tumour of mice treated with CUR blended with PVP. These last treated mice indicated a good anticancer activity and therapeutic effect. This data suggest that is possible to approach melanoma cancer through electrospinning technique.

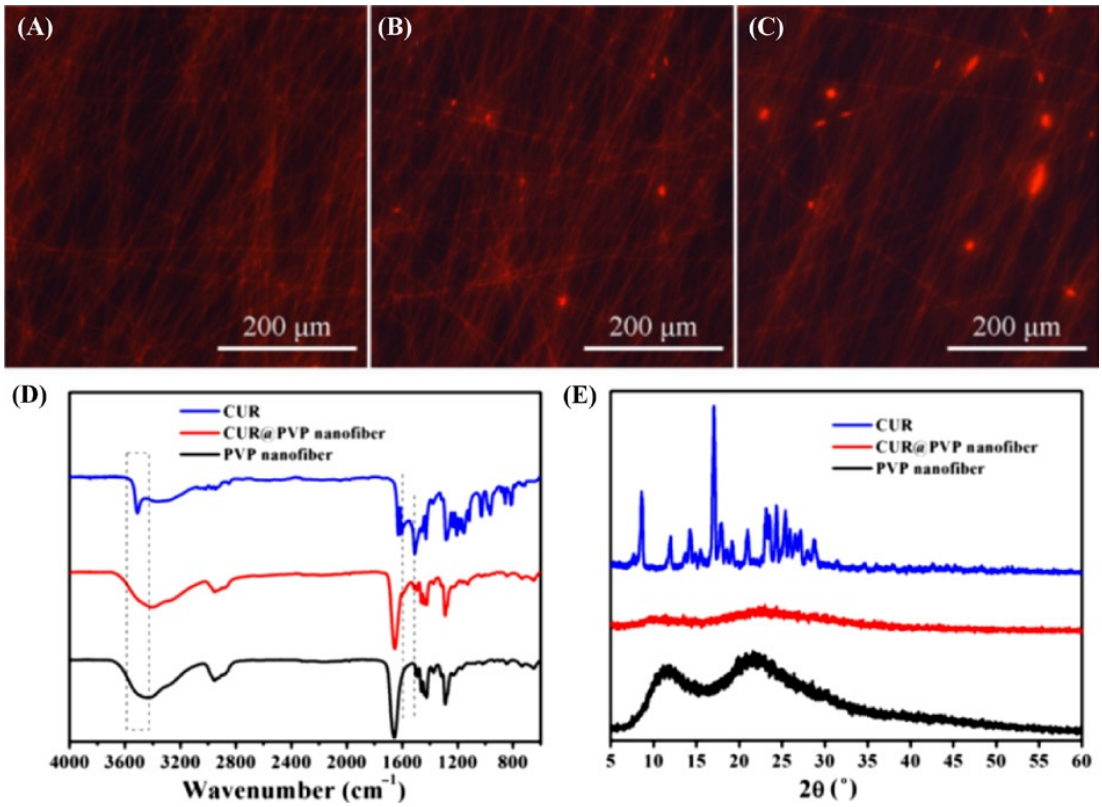

Figure 2-5 (A), (B), and (C) show the fluorescent images of CUR blended with PVP electrospun mats, 10%, 15%, and 20% wt% CUR, respectively. (D) shows FTIR spectra and (E) shows XRD data of nanofibers. *Source*: Open access doi: 10.1186/s11671-015-1146-2.

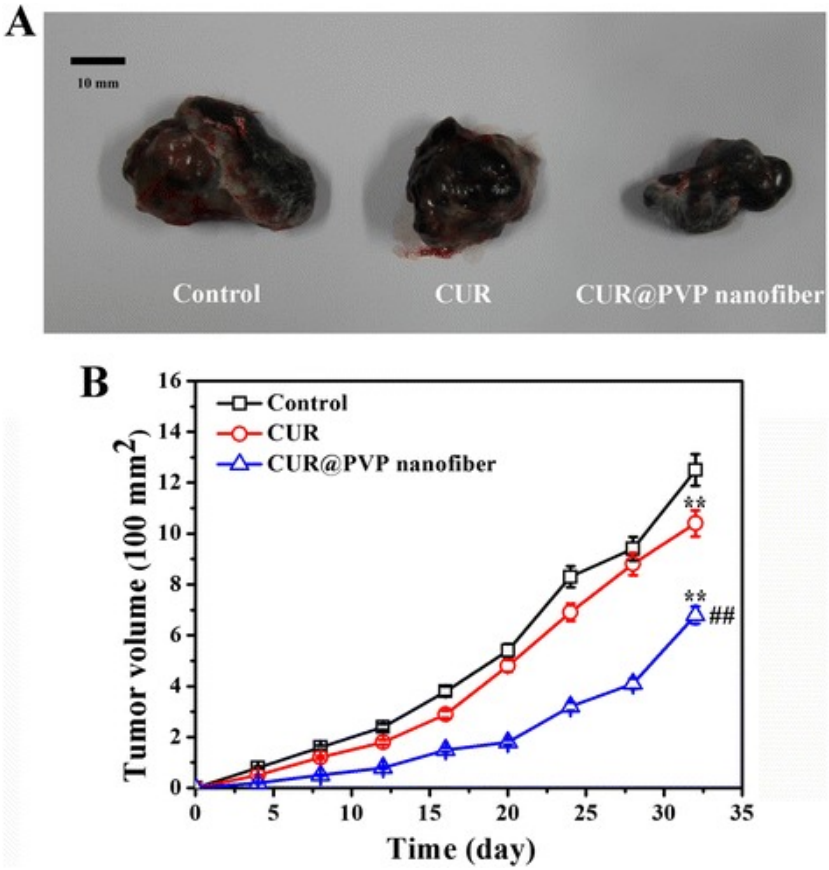

Figure 2-6 Digital photo of tumour with the treatment of different samples at day 32, (b) tumour volume vs. time profile. Values are mean ± SEM (n = 20). **denotes significant differences (P < 0.01) against control group, ## denotes significant difference (P < 0.01) against CUR group. Open access doi: 10.1186/s11671-015-1146-2.

2.1.4 Skin and Melanoma Microenvironment

Skin is the largest organ of our body. The normal skin consists of two distinct layers: the epidermis and dermis.

The epidermis is the upper layer formed by keratinocytes, melanocytes and Langerhans cells. Keratinocytes are the most abundant cell type in the epidermis layer; this type of cell is responsible to produce the major structural protein of the skin, many growth factors, and keratin to maintain the normal skin homeostasis. The keratinocytes cells also regulate and promote the proliferation of melanocytes through connexins, desmoglein-1 and E-cadherin (Li et al., 2003). The epidermal- melanocytes unit is formed normally by one melanocyte surrounded by 5-8 keratinocytes. Melanocytes cells are responsible to produce pigments called melanosomes containing melanin. The major risk of melanoma is the incidence and absorbance of ultraviolet (UV) radiation, upon UV exposure, the melanosomes are transferred from melanocytes to the keratinocytes, aiming to provide a mechanism that can avoid DNA

damages caused by UVR incidence (Kondo & Hearing, 2011). In the skin is possible to find cells that are an antigen, namely, Langerhans cells that are the dendritic immune cells.

The dermis is mostly formed by fibroblast cells, which are responsible for collagen production and release. In addition to fibroblast cells, we can find pericytes, adipocytes, macrophages and vascular endothelial cells. Fibroblast cells are also responsible to produce elastin, thus, maintaining thigh connection of the dermis and epidermis through a basement membrane.

Melanoma microenvironment conditions differ from the normal skin, the condition is altered as the melanoma phase is progressing. In the early stage of melanoma, it is possible to observe radial growth phase (RGP), followed by vertical growth phase (VGP), subsequently, the last phase which might worsen the patient´s condition occurs at the metastatic stage. When we are talking about melanoma microenvironment is important to mention that it is highly heterogeneous, once that contains a variety of extracellular matrix (ECM), noncancerous cells such, fibroblasts, keratinocytes and inflammatory cells, at this microenvironment it is also possible to find growth factor produced by stromal cells and cancer cells. The interaction between the cancer cells and the surrounding cells is very complex. The cytokines and growth factor produced by melanoma cells are responsible to recruit many types of stromal cells, activating the tumour microenvironment, which can be either indirectly or directly. The first case, when the growth is indirectly it affects many functions of other types of stromal cells, which may play an important factor in melanoma initiation, progression, and finally metastasis. When a tumour is activated directly, the melanoma growth is promoted by activate stromal cells within the tumour microenvironment (Wachsberger, Burd, & Dicker, 2003). **Figure 2-7**, we can observe murine melanoma cells B16.

Deprived of adequate oxygen supply, the hypoxia of dermal environment takes place, promoting melanomagenesis (Hockel et al., 1996). While the tumour grows, it is necessary to keep necessary nutrients, oxygen and blood supply, for this reason as a consequence, starts forming an uneven vasculature distribution (Wachsberger et al., 2003). Hypoxic tumour cells are often associated with worse prognosis, this kind of tumour is more resistant to radiation and chemotherapies, due to its peculiar biological properties and metabolic rates, once compared to well- oxygenated tumours (Harrison & Blackwell, 2004). The tumour aggressiveness and hypoxia are linked, in recent studies, researchers could demonstrate that tumours in the mouse when treated with antiangiogenic agents, can lead to tumour hypoxia, therefore, enhancing the invasion and migration properties (Bikfalvi et. al., 2011; Pàez-Ribes et

al., 2009). Tumour hypoxia and tumour- stromal cells microenvironment can play an important role in melanoma skin cancer initiation, progression, and treatment resistance.

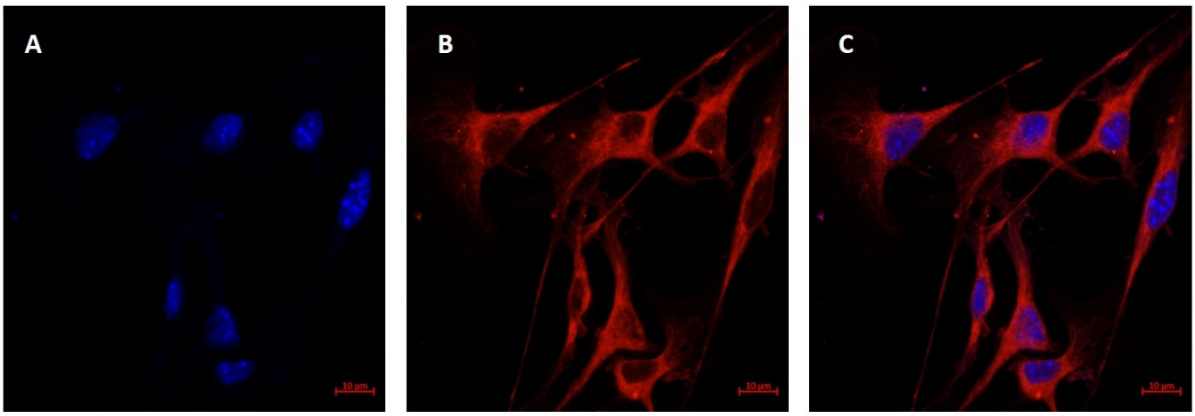

Figure 2-7 Confocal fluorescence images of B16 (murine melanoma cells). A-) Stained with Hoechst, to visualize the nuclei, blue; B-) cells stained with Phalloidin Rhodamine Actin, to visualize cellular morphology, red; C-) multiples layer. The confocal images were acquired by Zeiss LSM800 Airyscan microscope using x 63 oil immersion objective lens.

2.1.5 Human Dermal Fibroblasts (HDF) and Melanoma Association

The development of a melanoma tumour, the fibroblast cells can be activated by other types of stromal cells or tumour cells, playing an important role in tumour growth and progression. CAFs, are known as cancer-associated fibroblasts, which is highly heterogeneous in terms of their functions and markers. Commonly used in other cancers, the fibroblast-specific protein 1 (FSP1) is also expressed in melanoma cells (Andersen et al., 2004), for this reason it is possible to affirm that FSP1 is a good melanoma-associated fibroblast (MAF) marker, once that it is induced by melanoma cells, although it cannot be detected in normal skin fibroblasts (Huber et al., 2003). **Figure 2-8**, shows healthy human dermal fibroblast after 24, 48 and 72 hours incubation time.

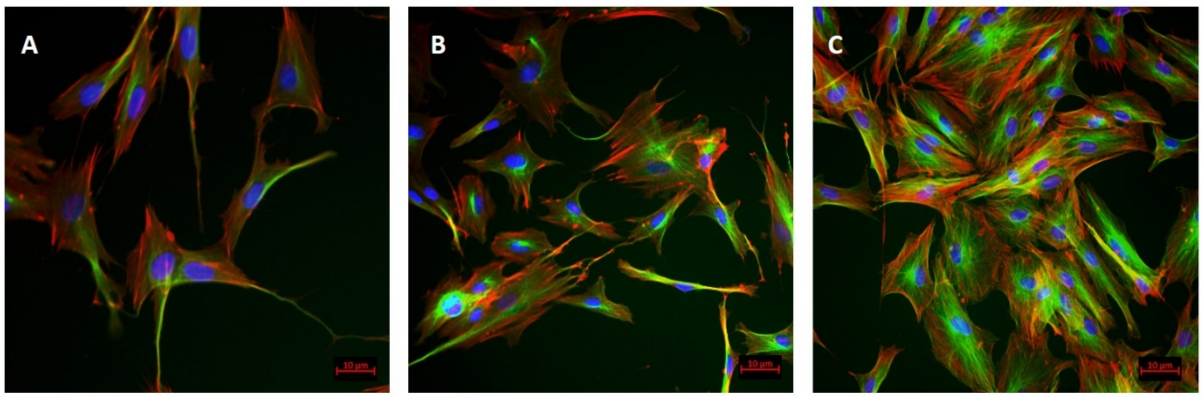

Figure 2-8 High Content Analysis of Human Dermal Fibroblasts. A, B and C represent 24, 48 and 72 hours after incubation time, respectively.

Particularly in the white population, skin cancer is by far the most common malignancy of humans. Cutaneous malignant melanoma (CMs) is one of the three commonest types of skin cancer, the other types of skin cancer include non- melanocytic skin cancer- NMSC such as squamous cell carcinoma (SCCs) and basal cell carcinomas (BCCs). The vascularization of a tumour happens naturally, the cells obtain all the required nutrients to growth till reaching the size range of $2mm^3$, this is called passive diffusion (Jones & Harris, 1998).

Melanoma cells growth and survival rates can be profoundly affected by MAFs. It was shown, in xenograft models, that co-injection of fibroblasts cells with melanoma cells increases the tumour growth (Gärtner, Wilson, & Dowdle, 1992). In another study, it was possible to observe that coculturing normal fibroblast cells in association with melanoma cells possibly promote the tumour growth, this growth has been reported in early-stage of the melanoma cells, on the other hand, it has a very little effect on melanoma metastatic cells (Cornil et al., 1991). Some fibroblast cells can promote growth factor by overexpression of insulin such as HGF, bFGF (Otsuka et al., 1998), and insulin growth factor-1 (IGF-1), leading to growth of biological early stages and inducing its survival rate of melanoma cells by activation of beta-catenin and mitogen-activated protein kinase (MAPK) (Satyamoorthy et. al., 2001). In addition to that, this growth factor also promotes vascular endothelial growth factor (VEGF) and epithelial growth factor (EGF), both are potent mitogens for melanoma cells growth, proliferation, and microenvironment. MAFs also promote the melanoma growth through glycosaminoglycan hyaluronan, which is also called as hyaluronate, hyaluronic acid or HA. The synthesis of HA (HAS1 and HAS2) in MAFs can activate and enhance melanoma production PDGF-CC and PDGF- AA, in dermal fibroblast, melanoma cell-derived factor stimulates hyaluronan synthesis by regulating HAS2 through p38 and PDGFR-PI3K-AKT signaling (Pasonen-Seppanen et al., 2012). HA is one of the major components of ECM and has been proved that HA can promote melanoma tumour growth, angiogenesis, and metastasis (Willenberg et. al., 2012).

The ADAM-9 is expressed at tumour- stromal border, it is a family of proteases with a disintegrin and metalloprotease domain (ADAM). Studies *in vivo* and *in vitro* denotes that stromal fibroblast- specific expression of ADAM-9 is responsible for melanoma proliferation and apoptosis, at the same time is controversial whether it exerts tumour growth inhibitory effect or pro- tumour growth (Abety et al., 2012; Guaiquil et al., 2009).

The gene p53 is related to several types of tumour, once that it can suppress the expression of the tumour suppressor (Bar et al., 2009). In stromal fibroblasts, the gene p53 can modulate the tumour growth in a

stromal cell-derived factor 1 (SDF1)- dependent manner (Addadi et al., 2010), dysfunction of p53 occurs in more than 90% of melanoma cases (Box & Terzian, 2008). In melanoma, MAFs may play an important role in metastatic effect, once that the invasive potential of human melanoma cells may alter the fibroblast gene expression (Bedogni & Powell, 2009).

The metastatic effect is caused by the spread of the primary cancer cells to distant sites of the body, causing most of cancer deaths. In melanoma metastasis is possible to observe sequential steps listed below (Nguyen, Bos, & Massague, 2009; Orgaz & Sanz-Moreno, 2013):

- Tumour cells are separate from the basement membrane, due acquisition of invasive phenotype;
- Tumour cells can reach either regional site or distant organs through hematogenous or lymphatic dissemination;
- Tumour cells survive in new metastatic organs such as bone, liver, brain, lungs, etc.

Fibroblasts cells may play an important role in melanoma angiogenesis, which consists in the formation of new blood vessels to supply nutrients for tumour growth, being one step away from tumour cell metastasis. In a three-dimensional gel embedded with fibroblast type I collagen model and melanoma cells, normal human fibroblast enable melanoma cells to induce angiogenesis (Goldstein et. al., 2005). MAFs can enhance melanoma invasion and angiogenesis, as consequence, promote melanoma metastasis.

2.1.6 Melanoma Initiation and Progression Through Hypoxia- Inducible Factors (HIF)

Hypoxia-inducible factors are present in solid tumours when the malignant tumour size rage is more than 1mm in diameter. As consequence, pockets of hypoxic regions can be visualized by immunohistochemistry with either CAIX (Pires et al., 2010) or pimonidazole (Post et al., 2004) or antibodies. Normal oxygen level in the skin ranges from 1.5 to 5% O_2. This concentration of oxygen is sufficient for stabilization of HIF-1α (Bedogni & Powell, 2006). In the dermal-epidermal junction, the melanocytes are physiological hypoxic, under normal conditions, thereupon, hypoxia may play an important role in melanocyte transformation. In normal skin, the nuclear HIF-1α is detected and suggests that HIF-1α is activated in melanocytes, in other words, HIF-1α is a microphthalmia-associated transcription factor (MITF) transcription target in melanocytes (Buscà et al., 2005). MITF plays a crucial role in melanoma cancer progression, once it is a transcription factor that is involved in the regulation of

genes which are related to migration, invasiveness, proliferation survival and metastasis of melanoma cells.

Known to induce tumour resistance to both radiation and chemotherapies, hypoxia has broad effects on tumour. Hypoxia encourages growth of cells with deficient function of tumour suppressor p53 (Lee et al., 2013), promotes genetic instability, reduces drug-induced apoptosis (Schnitzer et. al., 2006), and favors growth of hypoxia-tolerant tumour cells clones (Brahimi-Horn, Chiche, & Pouysségur, 2007).

HIFs may play an important role in melanoma tumour progression, chemotherapies resistance, and metastasis. Hypoxia can influence the metastatic progression, once it promotes a phenotype switch in melanoma cells from proliferative to invasive (Asnaghi et al., 2014). Proliferative melanoma cells once exposed to hypoxic microenvironment increase the invasive potential in a HIF-1α-dependent manner and downregulates melanocytic marker expression. Melanocytes are prone to oncogenic transformation under hypoxic microenvironment, due to HIF-1α stabilization. *In vivo,* under hypoxic condition, HIF-1α- deficient melanocytes show the ability to delay and diminished transformation capacity in tumour growth. Very aggressive melanoma can occur due to the expression of a nondegradable form of HIF-1α protein. A permissive environment of HIF-1α may act as a tumour promoter in cells, this activity is provided by enabling HIF-1α stabilization at low oxygen microenvironment in the skin, where tumour cells promoters have acquired oncogenic mutations and are genetically unstable.

2.1.7 Tumour–Associated Macrophages (TAMs)

The microenvironment of a melanoma tumour contains all types of inflammatory cells, including mast cells, neutrophils, dendritic cells, B and T cells, and macrophages. TAMs are the most abundant cell type among the immune cells, it can interfere directly to the tumour microenvironment by so many ways such as remodeling ECM, increase tumour initiation and growth, promoting angiogenesis and suppressing antitumour immunity through producing growth factors, cytokines, reactive oxygen and nitrogen species (NOS) and chemokines (Mantovani & Sica, 2010; Porta et al., 2007; Qian & Pollard, 2010). TAMs are also related to cancer resistance therapies as immune therapy, target therapy, radiotherapy, and chemotherapy. The patient's response to antitumour therapies can be predicted by the number of macrophages, which is a prognosis maker, the higher number of infiltrating macrophages the worse is the prognosis in a variety of cancers, including melanoma (Torisu et al., 2000). An inflammatory

microenvironment plays an important role in every step of melanoma development, it can be provided by TAMS and highly inflamed tumours.

The melanoma association with macrophages can be classified as (Wang & Yun, 2016):

- M1 Macrophages – Activated macrophages: the macrophage is polarized by pro-inflammatory factors as IFN-$_{\gamma}$ and microbial agents as bacterial lipopolysaccharide (LPS). In this macrophage, are produced reactive oxygen species (ROS), higher levels of nitric oxide (NO), TNF- α, IL-6, IL-12 and lower level of immunosuppressive cytokine IL-10. Many growth factors produced by M1 macrophages have a high effect on promoting tumour growth and metastasis such as IL-6 and TNF-α;
- M2 Macrophages – Alternatively activated macrophages: TAMs resemble M2 macrophages and exert pro- tumour activity. M2 macrophages produce lower levels of IL-12 a higher level of TGF-β, IL-1 receptor (IL-1ra), IL-10, CCL-1, CCL18, and CCL-22.

2.1.8 Melanoma Therapy by Macrophages Targeting

Many approaches have been developed to target macrophages in melanoma cancer, due to its multifunction and ability to promote tumour progression and metastasis, studying the mechanisms of macrophages on tumour development and the possibilities available to treat the patient´s tumour:

- *M-CSF Receptor Inhibitor:* M-CSF is the most potent growth factor which is highly expressed in melanoma cell lines. It is related to melanoma- associated macrophages activating multiples survival signaling for macrophages, once it binds to the M-CSF receptor (M-CSFR). Currently undergoing clinical trials, several M-CSFR inhibitors are being tested for metastatic solid and advanced cancers, including melanoma. These trials focused on cancer therapy from tumour microenvironment and cancer cells to tumour cells are summarized in **Table 2-2** . PLX3387, an M-CSFRi, was developed by Plexxikon Inc. It possesses the ability to increase the antitumour activity of BRAF[1] inhibitors. So, phase I is under way for patients diagnosed with melanoma. Anti-M-CSFR antibody, the AMG 820, from Amgen, are under trials for advanced solid tumour therapy. Also, used for treating a solid tumour, the M-CSFR inhibitor ARRAY-382 is also under clinical trial;

[1] BRAF is a human gene that encodes the protein called B-Raf, which is involved in sending signals inside the cells for cellular growth. In some human cancer this protein is mutated.

- *CCL-2 Inhibitor:* CCL-2 plays an important role in macrophage survival and differentiation. It is the most potent chemoattractant for macrophages. For melanoma therapy, some studies indicate that anticancer therapies in combination with CCL-2 targeting are useful and vital (Hansen et al., 2006). The expression CCL-2 can be decreased by the antitumour activity of BRAF inhibitors. *In de novo* tumourigenesis and mouse melanoma xenograft models can achieve the synergistic effect on tumour growth in combination therapy of an anti- CCL-2 antibody and BRAFi (Knight et. al., 2013);
- *Liposome- Based Assay:* composed by a bilayer of phospholipid, the liposome is an artificially prepared vesicle structure. The drugs can be phagocytosed and recognized by microphages when loaded in liposomes, providing an efficient macrophage targeting. The liposomes have less toxicity than regular chemotherapy agents and can minimize the degradation of chemotherapy agents in the serum (Kelly, Jefferies, & Cryan, 2011). Liposome- encapsulate clodronate (LIP-CLOD) sulfate, enable the macrophages apoptosis, the deletion of macrophages leads to the angiogenesis inhibition and melanoma growth in human melanoma xenograft model (Gazzaniga et al., 2007). In a study, reported by Banciu and co-workers, they stated that liposomal prednisolone phosphate has antitumour growth effect on deletion of TAMs in melanoma models (mouse B16) (Banciu et. al., 2008).

Table 2-2 Summary of drugs that target macrophages. Source adapted from: (Tao Wang , Sook Jung Yun, 2016).

Name	Clinical indication	Therapy Combination	Pathway	Phase
PLX3397	Advanced solid tumours	Paclitaxel	M-CSFR	I
	Unresectable or metastatic melanoma	Vemurafenib		I
	Gliobastoma	Termozolomide and radiation		I/II
AMG 820	Advanced solid tumours	NO	M-CSFR	I
ARRY 382	Advanced solid tumours	NO	M-CSFR	I

2.1.9 Skin Cancer Treatments

In the past decade, the therapeutic landscape has been rapidly changing regarding the process of skin cancer treatment. Target therapy, nowadays, is the hot topic, as an alternative to traditional cancer treatment such as chemotherapy and radiotherapy. Molecular- target drugs (e.g. MEK inhibitors, and BRAF inhibitors) and immune checkpoint agents (e.g. anti- PD-1, anti-CTLA-4), have been gradually taking

place of standard chemotherapy. The most used agent for chemotherapy treatment is the dacarbazine (DTIC).

The National Comprehensive Cancer Network (NCCN), in 2017, has done some recommendation for patients with or without BRAF mutations, having unresectable stage III or stage IV for melanoma. The first-line setting to approach this type of cancer include immunotherapy with checkpoint inhibitors nivolumab (category 1) or anti-PD-1 monotherapy with pembrolizumab (category 2A), or ipilimumab/ nivolumab combination therapy (category 2A). Cancers with category 1, the recommendations include MEK/BRAF inhibitors combination therapy with cobimetinib/vemurafenib or trametinib/dabrafenib or monotherapy with vemurafenib or dabrafenib targeting BRAF inhibitors. BRAF – target- therapy shows good efficiency in patients with metastatic disease and BRAF mutations. Patients diagnosed with PS 0 to 2 have multiple approaches, such as high dose of IL-2, BRAF/MEK inhibitors combination therapy if the patient has a BRAF mutation, chemotherapy, bio chemotherapy, anti-PD-1 monotherapy, ipilimumab/nivolumab combination therapy (Luo & Shen, 2017).

Apart from the above-mentioned approaches in this thesis, we will make a more detailed approach to the topic of chemotherapy and radiotherapy treatment focused on melanoma skin cancer. These therapies are reported as causing, in some cases, severe side effect, leading to patient´s damage or worsening health condition. Most of these treatments available, can have the apoptotic effect to malignancy and healthy cells at the same time. For this reason, this doctoral thesis is of a great advantage for the scientific knowledge, proposing the use of electrospun mats for topical application, working as a drug delivery system, at the same time provide skin regeneration and collagen release for the damaged surrounded area.

2.1.9.1 Chemotherapy

Chemotherapy treatment is reported as being the combination of one or several drugs, it can be such as aspirin or penicillin, it will depend upon what kind of disease the physicians intend to treat. However, most people, which are not used to the term chemotherapy, often link its name to cancer treatment. The use of any kind and dosage of drug is related and may vary in how they are taken, the chemical composition, the possible side effects and last but not least, their effectiveness to a specific type of cancer. Depending upon the disorder, the physicians may decide for either the use of single drug or combination of multiple treatments or drugs. Last decade, Schulmeister and colleagues (Schulmeister et al., 2005), used a pre-documented protocol, aiming to reduce error regarding the prescription or the treatment, so,

for this reason, all chemotherapy based treatments should follow and be provided on the basis of protocols.

Regarding the treatment of cancer cells, it is imperative a better understanding of the life cycle of a cell. It is known, that healthy cells have programmed and controlled lifecycle and carcinogenic cells cannot control, regulate their growth nor their interaction with the surrounding cells, thus, physicians may predict what is the best option for targeting a specific type of carcinogenic cells, if it is necessary the combination of multiple drugs, how often each drug should be administrated to the patients, and the dosage.

The American Cancer Society (American Cancer Society Staff, 2015), has done an explanation regarding the cell life cycle which is divided into five phases described below:

1. **G0 phase (resting state):** at this stage, the cell is not divided. Depending on the type of cell, G0 can last from few hours to a few days. Cells spend much of their lives in this phase. When the cells get signals to reproduce, it goes further into G1 phase.
2. **G1 phase:** this phase lasts about 8 to 30 hours. The cell starts making more protein, and, growing larger.
3. **S phase:** this phase lasts about 18 to 20 hours, the chromosomes containing the genetic code (DNA) are copied, so that, both new cells will match the DNA standards.
4. **G2 phase:** this phase comprehends from 2 to 10 hours, it is when the cell will check the DNA and get ready for starting to split into two new cells.
5. **M phase (mitosis):** this phase lasts only 30 to 60 minutes. The cell split at this stage into 2 new cells.

Chemotherapy is given to the patient through different pathways, depending upon the type of carcinogenic cells, most of the cases the patients receive the chemo dosage through the bloodstream or pills which can be taken. In some cancers, the chemotherapy is delivered directly to the patient´s body, where a tumour is located, aiming to deliver the drug directly to the affected body site and the same time, avoid the side effects. The side effects exist, especially when the drugs are taken through the bloodstream, once that the drugs can travel thought the body. As chemotherapy side effect we can mention: changes in mood or thinking, dehydration (lack of fluids), eating problems, falling, fatigue, fertility, fever, hair loss, hiccups, infection, leg cramps, low blood counts, lymphedema, mouth problems, nausea and vomiting, ostomies, pain, peripheral neuropathy, seizures, shortness of breath, skin problems, sleep problems,

stool or urine changes, sweating, swelling and weakness (American Cancer Society, 2017). The chemotherapy can be given to the patients through 3 main pathway (Naves & Almeida, 2015):

- Intralesional chemo: this kind of chemotherapy is often used for skin conditions, and for non-melanoma skin cancer such as basal cell carcinoma and squamous cell carcinoma. It is applied intralesional or via topical route, generally used for treating tumours locates under the skin, it is rarely used for cancers inside the body and when the surgery it is not the first option (Carrington, Stone, Koczwara, & Searle, 2010).
- Intra-arterial chemo: this type of chemotherapy is used, aiming to target more efficiently the tumour body site, concentrating the drugs in a tumour, thus, minimizing the systemic side effects. This method is one of the most used to treat cancer in several organs. The method is given to the patients by a flexible catheter (portable pump or attached as an implant) positioned right in the main artery that supplies blood to a tumour.
- Intracavitary chemo: it is related to the chemo which is given through a catheter, used to describe as chemo given right into a body cavity. This method as per se, is subdivide as: intravesical chemotherapy, intrapleural chemotherapy, intraperitoneal chemotherapy and intrathecal chemotherapy.

In 2002, Baguley (Baguley, 2002) in the study entitled *A brief history of cancer chemotherapy* stated:

> *"One of the most fascinating questions posed by dividing normal and cancer cells was the nature of the molecular clock that instructed the cell as to when it would replicate its DNA and when it would divide. Early studies in cancer tissue identified mitotic cells by their morphology and DNA- synthesizing (S- phase) cell by their uptake of tritium labeled thymidine (...) The first clues to the nature of the oscillator that ran the molecular clock were provided by the discovery in developing sea urchin eggs od a protein termed cyclin[2]. The cellular concentration of this protein increased up to the time of cell division and the abruptly decreased".*

2.1.9.2 Radiotherapy

Radiation therapy (RT) can be very beneficial when dealing with cutaneous malignancy. However, there are several oncologists and dermatologists whose have been hesitated to prescribe radiation therapy, due

[2] Cyclin is necessary to cell division.

to many issues and side effects. One of the biggest reason has been reported by Tward and co-workers (Tward et. al., 2012) that some of the patients who underwent RT treatment in their youth, treated with Grenz Rays[3], have gone to the development of some cutaneous malignancies later in their life. In RT, an electron beam energy should encompass a deep margin of a tumour by at least 90% distal, it is mandatory to choose an effective energy beam to achieve adequate surface dose. Mendenhall and colleagues (Mendenhall et. al., 1994) recommended for tumours smaller than 10 mm of a single radiation dose 20Gy[4], when the tumours are higher than 10mm is recommended the bean fraction, aiming to improve the therapeutically effect, minimize larger dose the tumour and avoid harmful radiation to the surrounding normal tissue (Lang & Maize, 2005).

Up to date, the RT is no longer used as it was used in the past, although, remains as an important alternative for cancer treatment, especially when dealing with metastatic melanoma (MM). It is reported in the literature that RT has been used as palliative treatment for MM (Gorayski et al., 2017).

The management of MM is highly complex due to its poor response to cytotoxic systemic treatment and clinical variables course. These poor cytotoxic include the use of fotemustine, dacarbazine, and temozolomide (Walker et. al., 2014). Other options that still cause significant toxicity and have a low response rate is the immunotherapy with ipilimumab and interleukin-2 (Alva et al., 2016; Menzies & Long, 2017).

The mutation test of tumour BRAF is a routine for all patients diagnosed with MM, thanks to the availability of agents targeting this intramolecular pathway. The overall survivals (OS) and prolongation of progression-free survival (PFS) are often linked to the use of BRAF inhibitors. The activation of BRAF protein kinase, once mutated, cause its activation in RAF/MEK/ERK pathway, therefore, stimulating cellular survival and proliferation (Dutriaux et al., 2015; Liszkay et al., 2014).

RT utilization rates for MM patients may vary considerably. The palliative radiotherapy (RT) is commonly utilized in patients with MM to treat symptoms as improve local control, avoid local progression and prevent the pathological fracture from bone metastases. Some estimative has been made, suggesting a

[3] Grenz rays are part of electromagnetic spectrum comprising low energy x-rays.

[4] The International Systems of Unit (SI) the Gray (symbol: Gy) is derived unit of ionizing radiation dose, which is defined as the absorption of one joule of radiation energy per one kilogram of matter.

rate around 21% of patients with established metastatic disease and 23% across all clinical stages (Lang & Maize, 2005).

Some physicians do not use RT as it was reported, that radiation has the tendency to develop atrophy and telangiectasias[5], as well the possibility of a further eruption of cancer over the time that might compromise the cosmetic appearance. In addition to that, RT may create a high risk for additional tumour formation, by damaging DNA of the cells (Gorlin, 1995).

2.2 Nanofibers Composites

2.2.1 Nanofibers Introduction

Nanofibers are widely used in various medical biomedical application such as tissue engineering, cell therapy, regenerative medicine, drug delivery and cancer therapy which is the main goal of this doctoral thesis. These fibers have a diameter range of 1 to 100 nanometers when the diameter is higher than 100 nanometers is called either electrospun mats or scaffolds developed by electrospinning technique. Thus, in terms of properties, scaffolds have proven to be much more efficient than any other system for molecular and cellular applications, as compared to their micro-macro scale. We can distinguish some functional properties, such as high aspect ratio, quantum confinement effects, large surface area, fast-absorbing biomolecules which provide abundant binding and adhesion to cell receptors. The strong cell-matrix interaction allows developing cell engineering, organs and tissues (Yang et. al., 2005). Electrospun scaffolds possess among all their characteristics surface morphology, mechanical strength, structural integrity, chemical functionalities and porosity, these characteristics can be tailored, accordingly to their employed application by modulating the fiber orientation, material composition, dimension and alignment. Monophasic electrospun mats have some numerous advantages, however, for some biomedical application is required composites scaffolds owing superior functional and structural properties (Ramalingam & Ramakrishna, 2017).

Several studies have been carried out on nanofibers composites, demonstrating that for biomedical and biological application, nanofibers composites are the better option than their monophasic nanofibers. When compared chitosan nanofibers to blended polycaprolactone (PCL)/chitosan, the cell proliferation

[5] Telangiectasis is also known as angioectasias or spider veins, which consists in small dilated blood vessels near the mucous membrane or the surface of the skin.

increased (> 50%) and obtained better mechanical properties to the composite nanofibers polycaprolactone (PCL)/chitosan than the monophasic chitosan nanofibers system (Prabhakaran et. al., 2008). Nanofibers composites are engineered materials composed of two or more distinct phase combined to impart new desirable, chemical, physical or even biological properties, processing bulk of different forms than those of any of the constituent phases. Ramalingam and Ramakrishna (2017) stated in Chapter 1:

> *"The matrix and reinforcing phases are the two components of the nanofiber composites. The matrix phase (also called continuous phase) is the primary phase, which is usually more ductile and less hard, and the reinforcing phase (also called secondary phase or dispersed phase) is embedded within the matrix, which is usually stronger than the matrix phase, as the name implies. A synergism produces material properties available from the individual constituent phases, while the wide variety of available polymer nanofibers and reinforcing phases allow for the choice of the optimum combination for the specific application".*

Statistical data shows the continuous development over the years for biomedical application research and development, as we can observe in **Figure 2-9**.

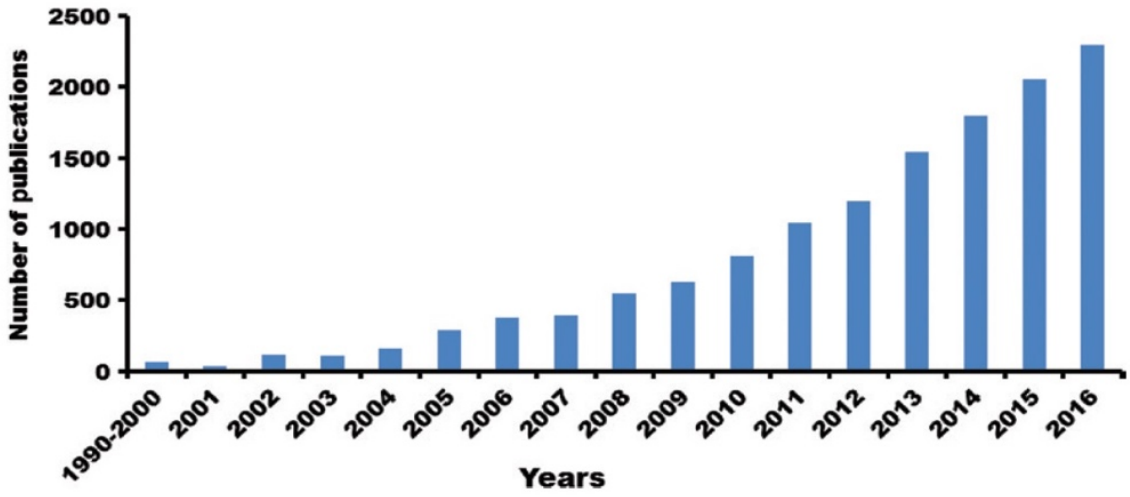

Figure 2-9 Statistical data representation for continuous growth of nanofibers composites in the field of biomedical research and development.

Nanofibers composites can be fabricated using various technique, such self- ensemble, phase separation, template synthesis and electrospinning. Among all these techniques, electrospinning appears to be the most widely used method, due to its ease fabrication, tunable structure and the possibility of large-scale fabrication. One of the most important characteristics is the resultant fiber is well suitable for a various

biomedical application. In **Figure 2-10**, we show the schematic representation of electrospinning apparatus.

The fabrication of nanofibers to develop scaffolds by electrospinning method is made by using an electrostatically driven jet of charged polymer melt or polymer solution (Murugan & Ramakrishna, 2007). Briefly, by applying an optimized electrical potential to the spinneret, the droplet located at the edge of the spinneret tip, polymer solution, gets electrified. There is an accumulation of charge on the surface of the droplet, which will be subsequently deformed into the Taylor cone. The droplet deformation is caused by:

1. Coulombic force, which is exerted by the external electrical field applied;
2. Electrostatic repulsion within the charge of the surface droplet and the Coulombic force.

The threshold occurs, when the electric field applied surpasses the critical value, the polymer electrostatic force at this stage tends to exceed the viscoelastic force and surface tension, consequently, is formed a very fine jet of charged polymer, which will be ejected from the tip of the Taylor cone.

As we can observe at **Figure 2-10**, the negative electrode is also called as fiber collector, it is the direction which the polymer jet will move towards. The instability zone is the distance from the Taylor cone and the collector of the fibers is where will happen a rapid evaporation of the solvents molecules. Due to the mutual repulsion, while in transit, the different polymers strands in the jet get separated, this phenomenon is known as splaying, rising to a series of ultra-fine dry fibers. The fibers are can be collected at the fiber collector (grounded metallic target), the range size may vary from nanometer to micrometers.

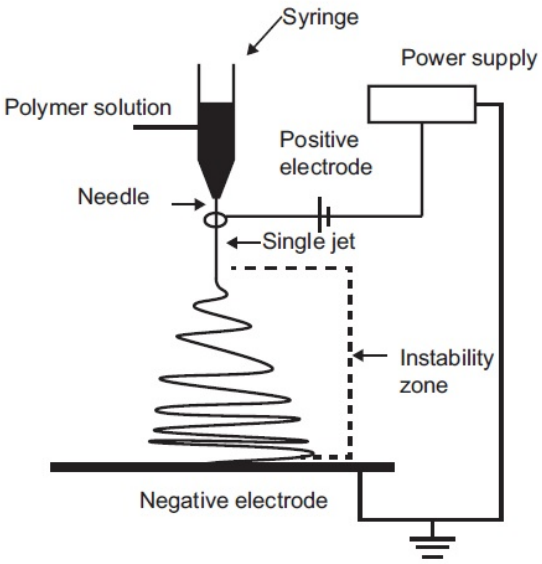

Figure 2-10 Schematic of electrospinning apparatus Source: (Wei et. al. 2011) adapted from p.119.

2.2.2 Nanofibers Composites Properties

Without compromising their volume fraction, nanofibers composites when compared to conventional composites, have been demonstrated to have a significantly large surface area. This large surface area can be useful to compensate the imperfection bonding between the fiber matrix interphases; therefore, even when prepared with the same volume fraction, nanofibers composites offer greater strength than conventional composites. These composites structures could be subject to surface either modification or treatment to impart or enhance new functional properties within them. In **Figure 2-11**, we show some of the key characteristics of the nanofibers composites, which are very tunable, it will basically depend upon the application and the specific need of the nanofibers composites. As an example, we can mention the poly (lactic-co-glycolic acid) (PLGA) nanofiber biodegradability which can be easily fine-tuned by just manipulating the concentrations of lactic or glycolic.

The interaction between the surrounding matrix and the nanofibers is one of the important factors that determine the composite properties interactions. These interface properties have been demonstrated to have the capability to control broadly properties, such as bonding strengths, bonding types and dislocation densities. The surface structure of each nanofiber will determine each nanofiber composites interface, therefore, every single electrospun mats surface will have a unique property (Ramalingam & Ramakrishna, 2017).

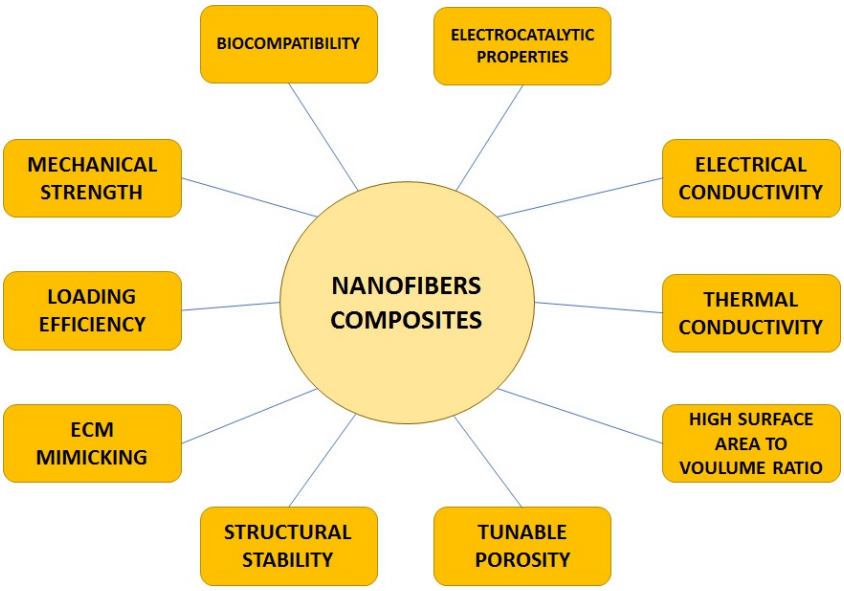

Figure 2-11 Schematic of the key nanofibers composites. Source: (Ramalingam & Ramakrishna, 2017), adapted from p.6.

2.2.3 Physicochemical Characterizations at Materials Level

One of the major features for characterization of electrospun mats is the fiber morphology, including diameter, alignment, shape (curved or straight; rounded or flat) and uniformity (uniform fibers of beaded).

The morphology of prepared electrospun nanofibers can be fast screened by optical or light microscopy, to observe a nanofiber composite or a thin layer of nanofibers. Even though light microscopy is more convenient, it cannot achieve the high level of resolution and magnification as an electron microscope can. The typical resolution of light microscope is about 200nm. In addition to that, by using the light microscope is not possible to visualize the three-dimensional shape of nanofibers. Therefore, most of the studies are made using SEM for morphological characterization of electrospun composites (Polini & Yang, 2017)

The SEM technique is described in the literature as a technique that uses electrons, instead of visible light to produce images of the samples. SEM has the wavelength much smaller than visible light, considering the electrons, for this reason, this technique enables high-quality images with resolution less than 1nm. In addition to that, the SEM technique has a good depth of field thanks to a small aperture and large work distance. The sample, when scanned by a focused beam of electrons, the various signals at the sample surface, which is produced by the interaction of the sample and the bean, can further be collected, revealing some important sample information such as surface topography, morphology, and chemical composition. The secondary electrons (SE), X-rays characteristics and backscattered electrons (BSE) are the most useful signal detected by SEM. The images of SEM can have the contrast property useful for the qualitative analysis of the sample, once that the contrast is dominated by the so-called edge effect: the secondary electron at the edge leads to increased brightness at the location which is being beamed. To reveal the chemical composition of nanofibers composites the BSE mode is the more used tool. The atoms with greater atomic numbers (larger atoms), due to their greater cross-sectional area, have a higher probability of producing an elastic collision, resulting in more BSE production (Polini & Yang, 2017). So, when analyzing electrospun composite fibers, the polymeric fiber matrix appears less bright than metal particles or doped ceramic (Zhang, Cong, & Yu, 2012). The SE images, generally, have higher resolution than BSE images, as the BSE is emitted from the depth of the sample.

Using SEM micrographs image analysis software, alignment and fiber diameter can be measured from microscopy images. The measurement of fiber diameters is made measuring multiple fibers of the

sample, the report result is described with ±, meaning the standard deviation, depicted as histograms of fiber diameter distribution (Ji et al., 2010).

2.2.4 Surface Modifications

The biofunctionalization of fibrous scaffolds is essential, the surface of electrospun fibers can be either incorporated with bioactive molecules or bioactive agents or even coated for use in advanced biological application with therapeutic properties. It is very important surface modification of the electrospun fibers for their use as antimicrobial or bioactive scaffold material (He et al., 2008; Kotaki et. al., 2005; Sadat, Amoabediny, & Ghaee, 2014; Song et. al., 2013; Wu et. al., 2010; Yao et. al., 2008).

There are many natural polymers that can be employed, having unique biological functions improving the characteristics during the fabrication of electrospun scaffolds. In some cases, the release of the drug of hydrophobic therapeutic drugs, can be directly blended into the electrospinning polymer solution, to fabricate the fibrous scaffolds. When using synthetic biodegradable polymers, they all are hydrophobic in nature, so, when developing electrospun scaffolds aiming cell adhesion to the surface of the scaffold, it is imperative the surface modification, changing the surface property from hydrophobic to hydrophilic, thus, enabling the cell adhesion, proliferation, and growth on the surface of the mats. Researchers stated (Baji & Mai, 2017):

> "*Typically, the hydrophilicity of the material can be improved by blending it with a hydrophilic polymer or by incorporating hydrophilic fillers within the matrix. However, a more straightforward technique is to improve the hydrophobicity of electrospun biodegradable polymer fiber using surface graft polymerization technique. Typically, plasma-induced polymerization can be used to graft polymer onto the surface of the fibers and modify the fiber surface chemistry and improve their hydrophilicity. This technique also introduces functional groups on the surface of the fibers that are used to bond covalently with bioactive molecules. The surface of the fibers is pretreated with UV irradiation to introduce polymer radicals to initiate graft polymerization of vinyl monomers".*

In a study of surface modification reported by Yao and colleagues (Yao et al., 2008), they used poly(4-vinyl-*N*-hexyl pyridinium bromide) to treat the polyurethane fibers. The polyurethane fibers were treated with the plasma treatment to produce peroxide and oxide groups to the mats surface, then, the fibers

were immersed in a solution of 4- vinyl pyridine monomer. Polyurethane fibers, were then, exposed to UV radiation, leading to the production of poly(4-vinyl pyridine), which were grafted into the polyurethane fibers. Followed these steps, the fibers were immersed into heptane solution containing hexyl bromide, thus, giving to the mats antimicrobial properties. In this study, the authors reported that after surface modification the fibers had excellent antimicrobial activity against Gram-negative *Escherichia coli* as well as Gram-positive *Staphylococcus aureus.* Therefore, these fibers might have a potential application on protective textiles, filters, and biomedical devices.

In a similar study, Ma and co-workers (Ma et al., 2005), used poly(methacrylic acid) as graft polymerization, to modify the surface of the polyethylene terephthalate (PET). Firstly, they treated the PET fibers with formaldehyde to introduce the hydroxyl functional group. The oxidation of hydroxyl groups using Ce (IV) formed free radicals. The following step was the grafting of polymerization of methacrylic acid. Finally, by using a coupling agent, poly (methacrylic acid) grafted PET is grafted with gelatin. They could show that gelatin is covalently attached to poly (methacrylic acid) grafted on PET fibers. In **Figure 2-12**, it is possible to observe the better endothelial cells (EC) growing on tissue culture plate **(A),** and better cell adhesion and proliferation in **(C)** than **(B)**, due to the surface modification of the electrospun mats by grafting gelatin, and **(D)**, the AFM images of the EC growing on the surface of the surface medicated mats.

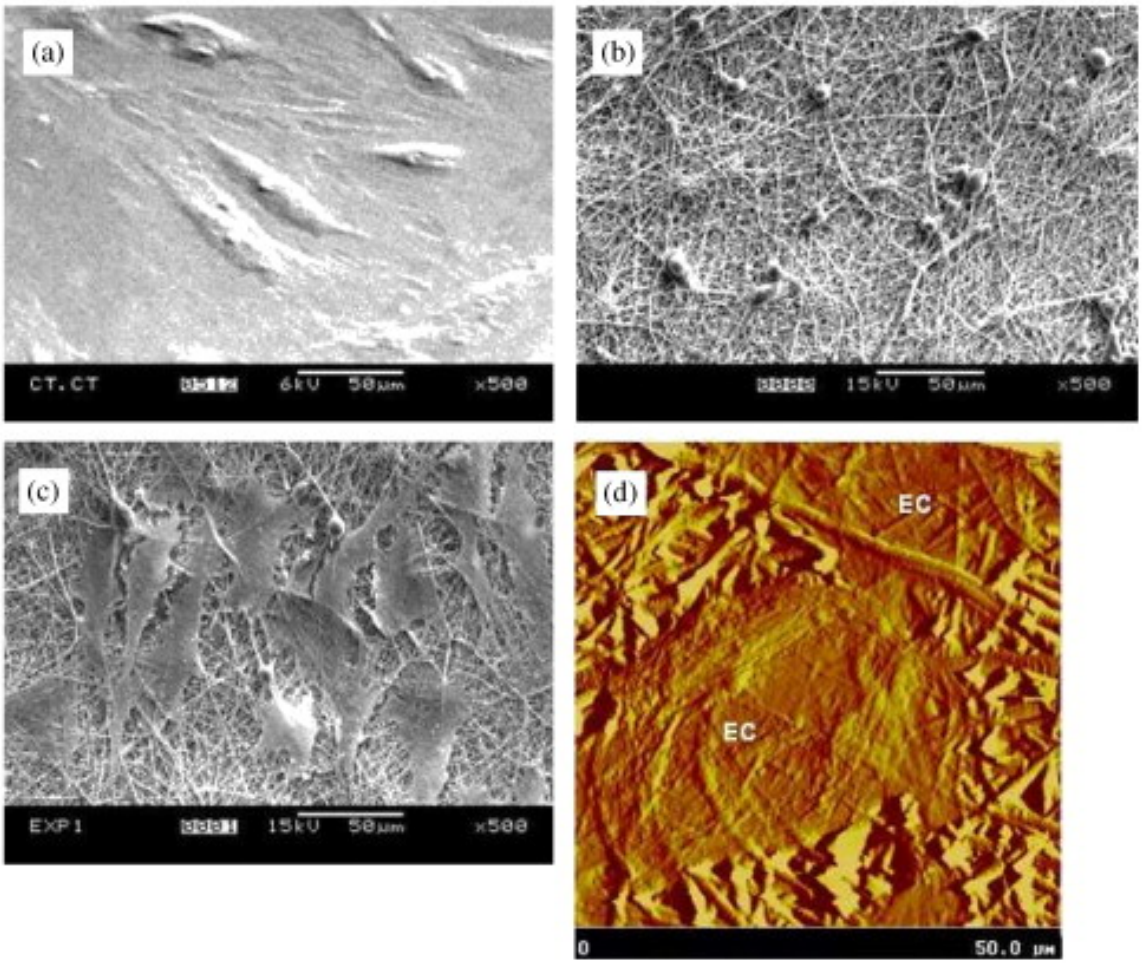

Figure 2-12 (a) Endothelial cells (ECs) cultured in TCP, SEM image, (b) pure PET NFM, (c) gelatin- grafted PET NFM, (d) ECs on the gelatin modified PET NFM- AFM image. Source: (Ma et al., 2005) p. 2533.

It was reported in another study (Song et al., 2013), that poly (lactic-co-glycolic acid) PLGA fibers could be reinforced by grafting small particles, by using poly (l- lactide) PLLA grafted on the surface of hydroxyapatite (HAP) nanoparticles for example. The presence of PLLA grafted on HAP increases the wettability of the fibers, and, at the same time, decrease the crystallinity of PLGA, consequently increasing the degradation rate. They reported that the bioactivity and the degradation of these composites fibers were dependent on the concentration of PLLA grafted HAP particles within the fibers. This surface modification was shown to promote greater cell adhesion and proliferation, once compared to the pure PLGA mats.

2.2.5 Hydrophilicity

One of the most influential parameters to control cell behavior via protein absorption is the wettability or surface hydrophilicity (Thevenot, Hu, & Tang, 2008). The most common method used to quantify the

wettability of a solid surface by a liquid is the water contact angle (WCA) measurement. This measurement is performed by a captive bubble or sessile drop technique in dynamic or static mode. Polini and Yang stated (Polini & Yang, 2017):

> *"Contact angle is defined geometrically as the angle formed by a liquid at the three phase boundary where a liquid (L), gas (G), and solid (S) intersect. The interfacial tensions, γSG, γSL, and γLG, form the equilibrium contact angle of wetting (ϑ), which is described by the well-known Young equation".* See **Figure 2-13**.

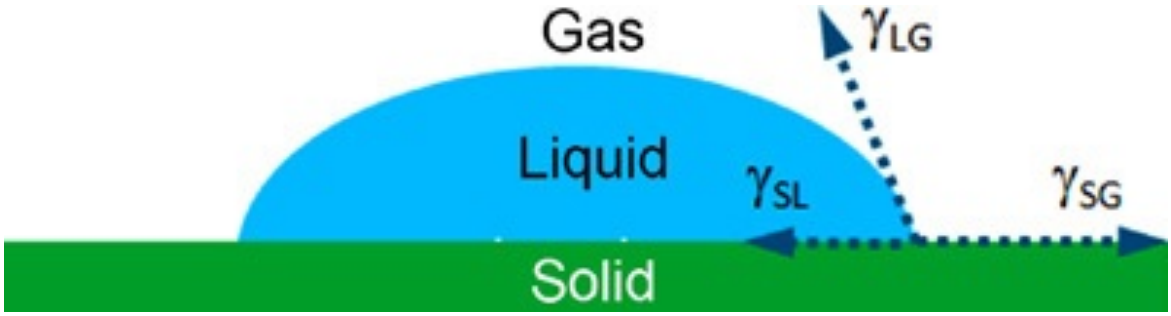

Figure 2-13 Young equation: γSG = γSL + γLG cos θ, where γSG is solid-gas surface tension, γSL solid-liquid surface tension, and γLG liquid-gas surface tension. Source: (Polini & Yang, 2017) p.107

The measurement of the contact angle is the most accepted method used to determine the degree of hydrophilicity/hydrophobicity of the given surface (Pasricha & Sachdev, 2017). High contact angle values indicate that the liquid cannot spread on the surface well while low contact angle values show greater spreading. If the contact angle is greater than 90 degrees, the surface is said to be nonwetting with the liquid. On the other hand, when the contact angle is lower than 90 degrees, the liquid wets the surface, by doing so, the surface has hydrophilic behavior. Hence, it is important to note that nanofibers composites and nanofibers surface is not flat, the texture on the surface can include the surface roughness and air- trapped pores, these characteristics together can directly influence on the measurement of contact angle (Onda, Schibuichi, Satoh, & Tsujii, 1996). For this reason, the result obtained by the wettability assay of nanofibers must be interpreted with great care (Bico, Thiele, & Quéré, 2002).

In addition to the morphological attributes, another factor that may contribute to the hydrophobic/ hydrophilic behavior of the nanofibers is its chemical composition, the heterogeneities, and surface structure, in addition to other minor factors (Pasricha & Sachdev, 2017). When the nanofibers are used

as a matrix or as scaffolds, the wettability of the surface influences, directly the interaction between living tissue and synthetic surface.

2.2.6 Mechanical Properties

The mechanical properties of materials are crucial for either the optimization of tissue engineering scaffolds or to design medical devices. The development of nanofibers, depending upon their application, may be required different mechanical properties. In the literature is reported that the measurement of the mechanical properties of single nanofibers is still extremely challenging, due to the difficulty in manipulating and isolating the individual nanofibers and measuring the deformation and very small force involved (Polini & Yang, 2017).

It is much easier to test the mechanical properties of bulk nanofibers composites of single nanofibers, due to the relatively large size involved. The mechanical properties of nanofibrous materials are strongly related to their fiber orientation and the porosity. A strong single nanofiber does not necessarily mean a strong nanofibrous membrane and vice versa. When measuring the mechanical properties of a nanofiber composite in membrane form, the specimens in dull-bell or rectangular shape are often used (Torres, Martinez & Lagaron, 2014; Yang et. al., 2009).

2.2.6.1 Stress-Strain Curve

Considered one of the most important tests to characterize the material's mechanical property response the stress-strain curve as the tensile test[6], which the specimen is clamped in a loading frame and subjected to a controlled displacement. Roylance stated (Roylance, 2001):

> "*As strain is increased, many materials eventually deviate from this linear proportionality, the point of departure being termed the proportional limit. This nonlinearity is usually associated with stress-induced "plastic" flow in the specimen. Here the material is undergoing a rearrangement of its internal molecular or microscopic structure, in which atoms are being moved to new equilibrium positions. This plasticity requires a mechanism for molecular mobility, which in crystalline materials can arise from*

[6] Stress- strain testing is detailed by standards- setting organizations as American Society for Testing Materials (ASTM). Tensile testing of composite materials is prescribed in ASTM D3039.

> *dislocation motion... Materials lacking this mobility, for instance by having internal microstructures that block dislocation motion, are usually brittle rather than ductile".*

When undergoing the tensile testing of the electrospun, it is obtained the stress-strain curve, which reaches the maximum stress right before the breaking point. In one study reported by Xu and colleagues (Xu et al., 2016), it was developed electrospun poly (ε-caprolactone)-pluronic- poly (ε-caprolactone)- based polyurethane nanofibers. In this study, they labeled the electrospun mates accordingly to the theoretical molecular weight of 3000, 6000, 8000, 10000, named PU3K, PU6K, PU8K, PU10K respectively. The fiber diameter obtained from the mats were, ~ 3.03 ± 0.99μm for PU3K, with the increase of molecular weight, the average diameter of the PU fibers significantly decreased. PU6K, PU8K, and PU10K had nanoscale fibers of 0.82 ± 0.36, 0.74± 0.19, and 0.71 ± 0.22 μm, respectively. The mechanical properties of electrospun PU scaffolds with different molecular weight were measured by the tensile test. In **Figure 2-14** shows the stress-strain curves of all samples, suggesting:

> *"PU3K scaffold had the smallest tensile strength at 0.82± 0.18 MPa and elongation rate at 117.5 ± 9.7%. Simultaneously, PU3K had the lowest Young's modulus of 0.78 ± 0.09 MPa, indicating that the scaffold was the most pliable. Along with the increase in the molecular weight of PU, both the tensile strength and elongation rate improved. PU6K scaffold had a tensile strength of 1.16 ± 0.25 MPa and an elongation of 171.7 ± 13.5%. With a further increase in molecular weight, although the tensile strength increased from 1.32 ± 0.30 MPa of PU8K to 1.63 ± 0.36 MPa of PU10K, the elongation rate suffered a loss, decreased from 165.5 ± 10.8% (PU8K) to 148.2 6 14.6% (PU10K). The Young's modulus of the four specimens showed the same tendency as the tensile strength; the PU scaffold with the highest molecular weight had the largest Young's modulus".*

A detailed explanation of Tensile Strength (MPa), elongation at break which is given in %, and Young´s module will be better explained in the next section.

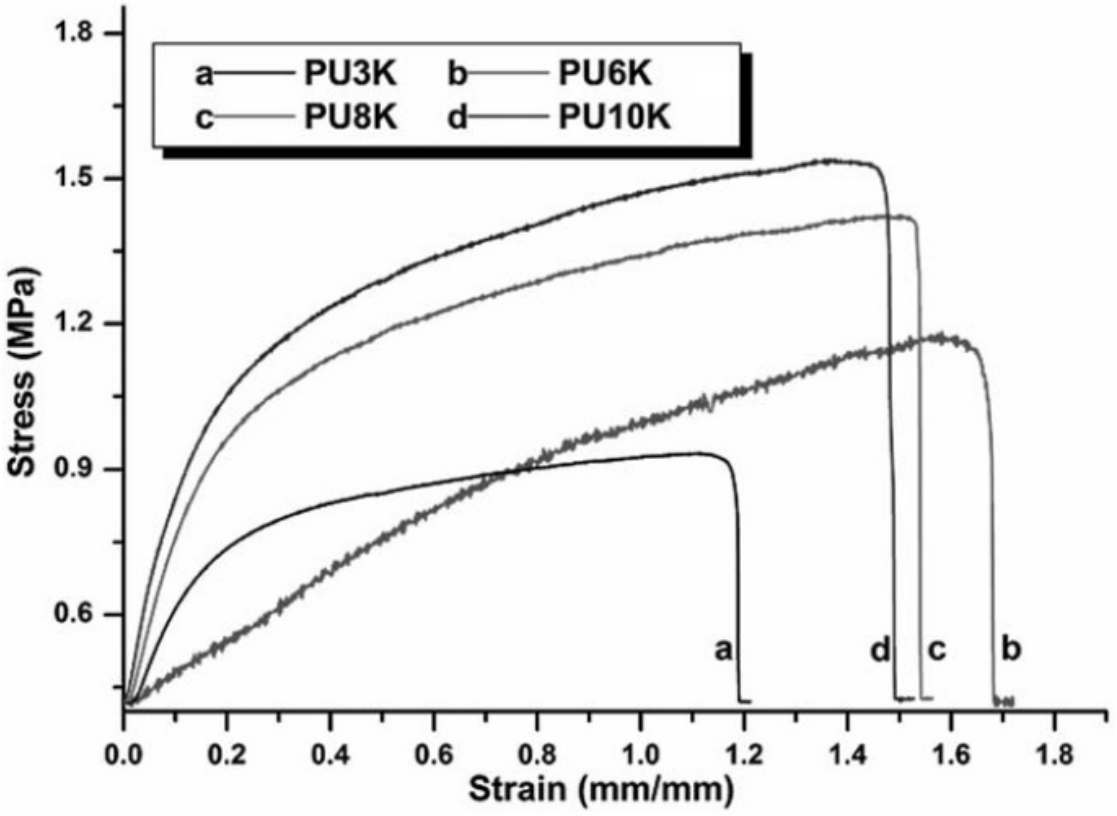

Figure 2-14 Strain–stress curves of the electrospun PU fibrous membranes, PU3K (a), PU6K (b), PU8K (c), and PU10K (d). Source: (Xu et al., 2016).

2.2.6.2 Tensile Strength

Polymers and polymer fibers consist of crystalline and amorphous domains. Under small tensile stress corresponding to the small strain polymer chain, found in the amorphous region, meant to be stretched (Sinha-Ray, Yarin, & Pourdeyhimi, 2014). The stretching force applied is followed by a response called entropic-response. Differently, as occurs in metals, in nanofibers mats even in the range of small stress and strains, when unloaded, as a result, occurs an irreversible strain, which remains (Khansari et. al., 2012). It means that even though the mats undergo a strain of 5%, the fiber mats will begin to deviate from the linear elastic response. As the fiber mats continue, it increases the probability of failure of the fiber-fiber junctions and individual fibers. When this behavior happens, the strength deteriorates dramatically and catastrophic failure is observed, as we can see in **Figure 2-14**, data showing the breakpoint of the PU3K electrospun mats at 1.2. Taking the study done by Xu and colleagues reported in the previous section we have mechanical properties data shown in **Table 2-3** for all the electrospun mats. The results reported by the authors suggested that the molecular weight of the PU copolymer significantly affect the mechanical properties of the PU nanofibrous mats. They also reported that the

higher molecular weight had the higher crystallization degree, so leading to a better mechanical strength, as we can observe in **Table 2-3**, the higher molecular weight of PU, results in a higher tensile strength.

Table 2-3 Mechanical properties of electrospun PCFC- Based PU Fibrous Membrane. Source: (Xu et al., 2016).

PU membranes	Tensile Strength (MPa)	Elongation at break (%)	Young´s Modulus (MPa)
PU3K	0.82 ±0.18	117.5 ± 9.7	0.78 ± 00.9
PU6K	1.16 ± 0.25	171.7 ± 13.5	0.96 ± 0.11
PU8K	1.32 ± 0.30	165.5 ± 10.8	1.15 ± 0.08
PU10K	1.63 ± 0.36	148.2 ± 14.6	1.28 ± 0.18

2.2.6.3 Young's Modulus - Modulus of Elasticity

Reported in the literature Young´s modulus is also called as modulus of elasticity, normally in a formula is referred as E, which basically, consist of the material property, describing its stiffness. Moreover, it is one of the most important properties of solid materials.

When a mechanical deformation is applied to the materials, these materials have energy. The energy is dissipated plastically or stored elastically. The way that the material stores this energy are summarized in stress-strain curves as in **Figure 2-14**. Consequently, the stress is defined as force per unit area and strain as contraction or elongation per unit length (Institute, 2005).

According to Hooke´s Law (the stress is directly proportional to the strain), we have the following equation (1):

$$\sigma = E \,.\, \varepsilon \qquad (1)$$

Where:

σ is the stress [MPa]

E is the modulus of elasticity [MPa]

ε is the strain [unitless or %]

Therefore, from the Hooke´s Law the modulus of elasticity is defined as the ratio of the stress train:

$$E = \frac{\sigma}{\varepsilon} \qquad (2)$$

In 2009, in a study made by Zhang (S. Zhang, 2009), it was reported a comparison between the aligned and randomly nanofibers, the effects of polymer concentration on the crystallinity of electrospun nanofibers, and later, the effect of the fiber orientation and diameter on the mechanical properties. Regarding the degree of crystallinity of the fibers, increasing the polymer concentration and the feed rate of electrospinning process, it decreases the electrospun crystallinity. In the solutions with lower concentrations, there are fewer polymer chains, as a result, there are fewer chain entanglements, which helps the crystals to be formed. The lower polymer concentration, the higher the degree of crystallinity.

The feed rate of electrospinning process can explain its correlation to the crystallinity degree. The complete cycle from the tip of the needle to the grounded collector is relatively long when the feed rate is lower. The crystallinity is increased, when the polymers can whip more completely comparing to higher concentration, thus they are stretched more than polymers with a higher feed rate. The alignment of the fibers does not change the crystallinity. Zhang stated:

> *"The aligned fibrous mats processed much higher Young's modulus and tensile strength than unaligned fibrous mats. This is due to that these two forms of electrospun nanofibers have characteristically different responses to strain resulting from two breaking mechanisms: the nonwoven mats were broken due to the failure of junctions and cohesion among the fibers at the bonding sites; however, the aligned nanofibers comprising the mat were oriented in the direction of stretching, thus mats were broken when the individual fibers were broken (...) The mechanical data also revealed that for aligned fibers as fiber diameter increases, Young's modulus also increases. While theoretically Young's modulus should be independent of fiber diameter. There are two reasons: firstly, to have the same cross-sectional area, the higher number of smaller fibers is required and this would increase the chance of fiber having defects which cause the fibers easier to be broken. Secondly, the alignment is not perfect. A higher number of smaller fibers has more chances to be unoriented with the direction of strain, thus they have lower Young's modulus compared to larger fibers (...) higher degree of crystallinity leads to lower Young's modulus for the aligned nanofibers. Because higher crystallinity means polymer chains are more oriented which results in the smaller diameter, as discussed above, with a smaller diameter, fibers have more chance to have defects, which makes them easier to break and the modulus was lost during crosshead displacement and results in lower modulus".* (Zhang, 2009)

We can conclude that the statement made by Zhang, when he says, *"While theoretically Young's modulus should be independent on fiber diameter...",* once compared to Young´s modulus data from **Table 2-3**, is true, once that in the study made by Xu and coworkers, as they increase the molecular weight of the polymer solution, the diameter of the fibers was decreased, however, Young´s modulus was increased.

2.2.6.4 Elongation Strength

According to the IPC- TM- 650 Test Methods Manual (Circuits, 2006), the percent of elongation is calculated by dividing the elongation of the material at the moment of its rupture by the initial gauge length and multiplying by 100. When gauge marks are used to define a specific test section, only this length is used in the calculation. If the gauge is not used, then the distance between the grips is used as the initial gauge length. The result of elongation strength is always given in percentage as we can see in the following formula:

$$Percent\ elongation = \frac{(elongation\ at\ rupture) \times 100}{(initial\ gage\ length)} \qquad (3)$$

In **Figure 2-15** is possible to observe the comparison between the strength applied to the material versus the elongation. Note that different types of materials have different rupture point. According to the image is possible to observe that composites and polymers can undergo from middle to strong strength application and are ductile regarding their elongation. The elongation measures the percentage change in length before the fracture, as per se, the elongation to fracture is a measure of ductility.

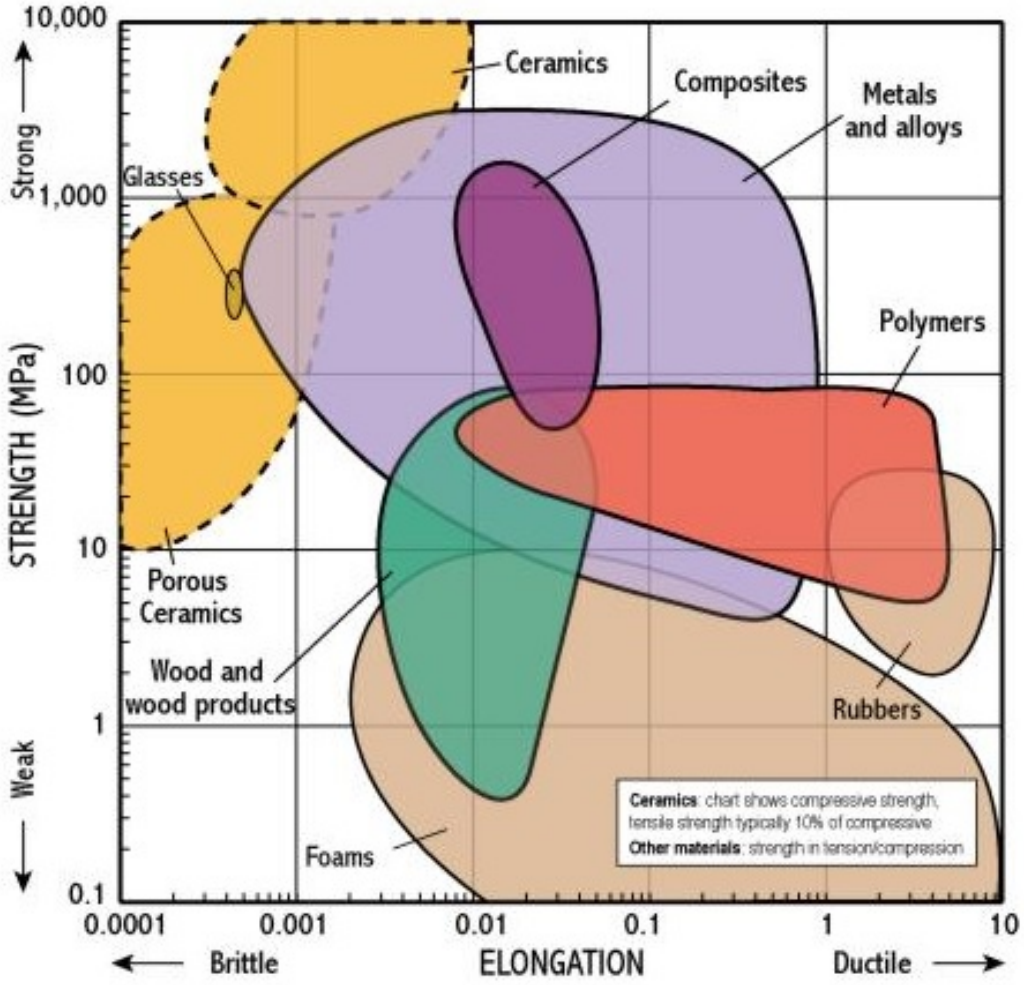

Figure 2-15 Stress versus elongation for different materials such as composites, polymers, rubbers, foam, porous ceramics, glasses, metals and alloys, and, wood and wood products. Source: ("Strength - Elongation," 2015).

2.2.7 Porous Structure

In nanofibrous materials employed as drug delivery systems and tissue engineering scaffolds, the pore structure of these materials is crucial. Generally, the pores of nanofibrous materials are formed by the intersection of nanofiber, it has been reported in the literature that some surface of single nanofiber itself may also possess porosity (Zhou et al., 2015). The most common techniques to evaluate the pore include mercury intrusion, BET (gas absorption- Brunauer- Emmett- Teller method) and gravimetrical method, although the most used technique to characterize the porous nanofibrous membranes are pore size distribution, specific surface area, average pore size and porosity (Polini & Yang, 2017).

Mercury intrusion porosimetry is used to characterize the porosity- related characteristics materials. The first step consists in weighting the specimens, then the specimens are placed into the chamber which

will further have the void space filled with mercury. An external force should be applied in order to make the mercury to penetrate the pores, once that mercury is a nonwetting agent for most of the substances. The Wasburn equation (Zhou et al., 2015) give us the pore diameter, where the required equilibrate pressure is inversely proportional to the range of the pore diameters, and to the size of the pores. The technique of mercury intrusion porosimetry may deform most of the electrospun materials, due to high-pressure application required, which may lead to an underestimation of porosity, the smaller the pore size the higher compression pressure application required. For this reason, this method is more used to measure pores down to a few microns.

$$d_p = \frac{-4\gamma cos\theta}{P} \quad (4)$$

Where:

d_p is the pore diameter

γ is the surface tension of mercury

θ is the angle between mercury and the sample

P is the pressure.

BET is mostly used to characterize the porous structure by physical absorption of a gas (e.g., nitrogen) in the specific surface area of specimens then calculating the amount of gas absorbed to a monomolecular layer on the surface of the nanofibrous material (Khajavi & Abbasipour, 2012). The BET technique is combined, in most of the cases, with Barrett- Joyner- Halenda (BJH) analysis, aiming the determination of the pore distribution.

Gravimetric measurements, the mass and the volume of the sample are first measured to determine the apparent density ρ_{ap} (Pham, Sharma, & Mikos, 2006). Then according to the equation, the porosity is calculated:

$$Porosity = \left(1 - \frac{\rho ap}{\rho m}\right) \times 100\% \quad (5)$$

Where:

ρ_{ap} is the apparent density

ρ_m is the density of the bulk material

Some composites materials are based the ρ_m on their respective densities and the weight ratio of the components (Zhou et al., 2015). Even though the gravimetric measurement is a convenient technique for porosity measurement, it is not applicable to the measurement of a specific surface area nor pore size (Polini & Yang, 2017).

2.2.8 Morphology

The characterization of electrospun are the major features of the fiber morphology, including alignment, shape (straight or curved; the round of flat), diameter and uniformity (uniform or beaded fibers).

To observe a thin layer of nanofibers or nanofibers composites, usually, the fastest way to screen the morphology of prepared electrospun nanofibers is through the optical or light microscope. However, the most used technique used for morphological characterization of nanofibers composite is SEM, once that light microscope cannot achieve the level of resolution and magnification as an electron microscope can. Normally the typical resolution of the light microscope is about 200nm (Polini & Yang, 2017).

The SEM microscope uses electrons beams instead of light to produce images of a sample. By doing so, the SEM has a significant potential to provide images with high quality with a resolution less than 1nm, once that the electrons have the wavelength much smaller than visible light. One of the great features of using SEM microscope is the possibility of revealing the three-dimensional shape of nanofibers because it has good depth of field thanks to the small aperture and large working distance. In this type of microscope, the sample is scanned by focusing the beam of electrons, the interaction by electrons and the sample, produces various signals at the sample surface, enabling the capture of these signals, revealing important information about chemical composition, surface topography, and morphology. The most useful signals detected by SEM apparatus are secondary electron (SE) and backscattered (BSE), they can be described as (Zhang et al., 2012) :

- Secondary electron (SE): is dominated by edge effect, where the SE at the edge of the samples can leave the sample more easily than the internal areas;
- Backscattered (BSE): this is a useful tool for revealing the differences in chemical composition of nanofibers composites. The BSE can have more production, when analyzing atoms with a greater atomic number, having a higher probability of producing an elastic collision due to their greater cross-section area.

2.2.9 FDA Approved Composites

The U.S. Food & Drug Administration (FDA), is an agency within the Department of Health and Human Services.

> *"The FDA's organization consists of the Office of the Commissioner and four directorates overseeing the core functions of the agency: Medical Products and Tobacco, Foods, Global Regulatory Operations and Policy, and Operations"*
>
> (F. Organization, 2017).

The FDA is responsible to validate and characterize either the drug is safe and can be used for any medical device or biomedical applications. The biomaterials are defined as any natural or synthetic substance engineered to interact with biological systems to direct medical treatment.

All biomaterials must be biocompatible, meaning that they should perform their function with desirable and appropriate host response. When designing a biodegradable biomaterial, it is necessary to take into consideration some important features such as (Ulery, Nair, & Laurencin, 2011):

1. Possess degradation time compatible with their function;
2. Do not cause sustained inflammatory response or cytotoxicity to the living tissue;
3. Produce nontoxic degradation effect that can be readily either resorbed or excreted;
4. Include appropriate permeability and processability for the designed application;
5. Have appropriate mechanical properties for their application.

Polymers have been used in the last few decades by the scientific community, to achieve a better development of efficient drugs, targeting the need and minimizing the side effects. They have a significant potential due to the flexibility in chemistry gives rise to materials with a great diversity of mechanical and physical properties. The polymers with greater degradation, have more attention on the process of developing new drugs and medical devices once that they are able to excrete, breakdown or be resorbed without the need of invasive approaches such as surgical intervention or removal.

Among all applications of biomaterials made from composites, we can distinguish the drug delivery devices and tissue engineering. The host response of these composites biomaterials may vary according to the physical, biological and chemical properties of the biomaterials. It is important to note that when these materials are at the same time biodegradable, there will be always an additional issue regarding this matter, which consist on the continued changes in the material properties induced by degradation

over the time. The degradation of these materials may lead to a long-term host response, differently than the initial response. These issues have contributed to the evolution, of biodegradable polymeric as a field of research.

The development of new composite materials, application of polymers for drug delivery systems, as well, for the development of new biomaterials have been growing thanks to the efforts done by researchers worldly wide, combining different expertise in materials, biology, chemistry, engineering and chemical practices (Ulery et al., 2011).

2.2.9.1 Polycaprolactone (PCL)

Poly(ε-caprolactone), also known as (PCL), is a biodegradable and biocompatible polymer (Gazzarri et al., 2013), which has slow degradation rate and excellent mechanical properties (Luong-Van et. al. 2007; Merrell et al., 2009; Selcan et. al., 2014). Since the 1970s, it has been approved by US Food and Drug Administration (FDA) as a safe component in implants. Thereafter, is has been frequently used in biomedical applications. PCL has been used in a variety of application, such as bone tissue engineering, packaging materials and implantable biomaterials due to its slow degradation nature (Luong-Van et al., 2007; Sheikh et al., 2014).

The application of PCL in tissue engineering application may have some limitation, once the polymer possesses poor cell affinity due to its hydrophobic nature. Plasma treatment can overcome this limitation and improve the hydrophilic effect of the material (Shin et al., 2010). The high molecular weight PCL impart high elasticity and tensile strength, making it attractive in the manufacture of bone, cartilage, suture, tendons and other medical applications, where mechanical strength is a major requirement. When dealing with electrospun technique, PCL has some disadvantages as lack of cell adhesion, intrinsic hydrophobicity, and absence of biological recognition site (Sheikh et al., 2014).

Among the variety of biomaterials that have been investigated over the last few years for tissue engineering, drug delivery systems and skin grafts, electrospun nanofibers with high porosity and high surface-to-volume ration can increase the efficiency of cell attachment, it has attracted the great interest of PCL use for the biomedical application. In addition to that, the loading capacity of nanofibers can be increased by the porous structure, becoming a great candidate for immobilization of biomolecules (Polat et. al., 2015). The latter may be remedied through internal modification of PCL nanofibers to counteract hydrophobicity, to incorporate hydrophilic constituents, and to enhance biocompatibility. Some attempts

have been made to approach this strategy include blending PCL with some natural bioactive polymers, such as gelatin, chitosan, collagen, cellulose, lecithin were used as an alternative to improve interaction between PCL scaffolds surface and cultured cells (da Silva et al., 2009; Zhang et al., 2012; Zhang et. al., 2005; Zoppe, Peresin et. al., 2009). Also, chemical modification of PCL nanofibers by grafting with hydrophilic dextran, polyethyleneimine, cellulose nanocrystals and poly(ethylene glycol) has been performed to improve cell proliferation and cell spreading on the surface of the electrospun mats (Ji & Hyuk, 2007; Kim et al., 2009; Zoppe et al., 2009)

2.2.9.2 Linear (LPEI) and Branched (BPEI) Polyethylenimine

It is known that cancer cell lysosomes may play an important role in intrinsic multidrug resistance (MDR), which is an important hindrance to effective cancer therapy by accumulating chemotherapy drugs and deactivating their therapeutic action. The polymer studied in this manuscript, the cationic charged organic macromolecule which contains amino nitrogen as every third atom, polyethyleneimine (PEI), can disrupt the lysosomal/endosomal membrane via proton-sponge effect (PSE) to provide an effective buffering system for the sudden decrease in pH from the extracellular environment to the endolysosomal compartment (Huh et al., 2007; Zhou, Murdoch, & Shen, 2015). Therefore (PEI) has been used to transfect a variety of cell types, both *in vitro* and *in vivo* (Reed et. al., 2006). PEI is available in both branched (BPEI) and linear forms (LPEI), they belong to the class of synthetic cationic polymer with excellent transfection efficiency with antiviral properties and gene delivery (Fox et al., 2016; Parhamifar et. al., 2010; Spoden et al., 2012).

A number of quaternized derivatives of PEIs, either in form of coatings or nanoparticles have shown to elicit potent antimicrobial activity against Gram-negative and Gram-positive pathogens. However, there exist only a limited number of studies that have evaluated the cytotoxicity of PEIs for melanoma cells. Linear or branched PEIs cytotoxicity is directly related to the polymer molecular weight and exposure time. The use of PEIs for biomedical applications is attractive because PEI-coated medical devices are under medical trials for extracorporeal blood purification therapies and PEI-based hydrogels have been approved by the US Food and Drug Administration (FDA) (Fox et al., 2016).

Scientists have done several approaches for melanoma cancer treatment and gene therapy including both PEI polymers, either Linear or branched (Ogris et al., 2003), given by their attractive properties for drugs delivery systems (Seong-Cheol et. al., 2013). PEIs are promising synthetic vector for drug or gene delivery due to its high cationicity, allowing it to protect and condense small interfering RNA or nucleic

acids (plasmid DNA), enabling proton sponge effect by endosomal escape and well nuclease digestion (Benjaminsen et. al., 2013). The kinetics of cells association of both, is very important, once that there is the substantial internalization of PEI bound to the plasma membrane of malignancy cells. However, some non-specific binding could be expected due to the ionic interaction, the polycations interactions with the components of the cellular membranes, may regulate their cellular uptake route (Seib, Jones, & Duncan, 2007). The toxicities and transfection efficiencies of PEIs differ in structure dependent or mass dependent manner, so far it is very difficult to have any defined relationship between these parameters (Han et. al., 2014; Kwok & Hart, 2011). The use of PEIs might be limited by its proinflammatory effects and high toxicity effects (Kawakami et. al., 2006).

2.3 Biomedical Applications of Nanofibers Composites

2.3.1 Drugs Delivery Systems

Recent advantages in the nanotechnology field have enabled the development of new alternatives to approach the treatment of several skin diseases, this approach aims to design a more efficient and controlled drug delivery systems, and at the same time minimize the side- effects of the traditional drugs, in which more often are administrated orally or through intravenous pathway. Drugs designed to deliver active compounds are thought to not interfere directly with the metabolism of the patients, therefore, they are a less invasive than traditional approaches (Naves et al., 2017c). Nanotechnology refers to the fabrication, characterization, and applications of active substances for various use (Sridhar et al., 2015). When designing a new drug delivery system (DDS) it is very important to take into consideration some major primary criteria such as the therapeutic window and the desired drug dosage. Drugs delivery nanocarriers are possible by employing based nanomaterials. With nanotechnology and the use of nanomaterials together, it becomes possible to encapsulate a variety of important therapeutic agents, such as peptide protein-based drugs, nucleic acid, and small molecules either hydrophilic or hydrophobic, minimizing the toxicity in healthy cells and at the same time helping to enhance the therapeutic bioavailability at the target area. By encapsulating different chemicals and molecules into the nanocarriers it is possible to monitor the solubility and the stability of drugs (Langer, 1998). Biological nanomaterials are preferable when possessing some important characteristics such as biodegradable, non- toxic, biocompatible, chemical compatibility with physiological solutions, preferably the use of biological/natural materials, easy to modify and design (Korrapati et al., 2015).

Many functions in the skin are performed by the stratum corneum. It plays a major role in the regulation of the transport of different chemicals compounds into the skin. Therefore, the skin is the largest organ in our body that can be approached to delivery active compounds or different drugs by transdermal or topical route. Passive and active skin penetration has been achieved and improved the efficiency of either topical delivery (the drug is delivered to the skin strata) or transdermal delivery (the drug is delivered into subcutaneous tissue, and later, taken up systemically into the body. The new topical therapies have gained over the last few decades a huge interest of the scientific community, once that it can be used as a tool to treat several types of skin disorder as psoriasis, contact dermatitis, and skin cancer which is the aim of this Ph.D. thesis. These drugs are delivered directly into skin strata (Zheng et. al., 2014). Over other delivery routes topical and transdermal systems ensure additional vantages, among all, we can distinguish: maintain the therapeutic effect of the drug at a lower dosage, enhance the biocompatibility by evading the hepatic first-pass metabolism, and bioavailability of the therapeutic agent at the target cells or tissue. The point of prime concern is the drug penetration while adopting the transdermal drug delivery route. It has been reported, in this context, that nanoparticles can enhance the drug penetration efficiency across the mucous membrane and the skin barrier (Zheng et al., 2012).

In 2017, Naves and co-workers stated (Naves et al., 2017c):

> *"Transdermal drug delivery system is an important route to deliver drugs into the body; the delivery through this approach is focusing in many alternatives to overcome some crucial problems related to the protective barrier of the skin. This approach may offer several advantages; since the skin is the biggest organ in the human body, it represents a relatively and readily accessible surface area for drugs absorption. The delivery of medication through transdermal route is less invasive when compared to other approaches, such as intravenous and oral route; this last approach can lead to drug degradation under extreme acidity of the stomach, and might interact with food, causing erratic delivery".*

In addition to the above statement, we can also mention that transdermal route allows ceasing the compounds or the absorption of the drug, preventing undesired effects and in the worst scenario drug overdose (Contreras, 2007). However, this method also presents some disadvantages, once that not all compounds available are suitable as nanocarriers to overcome the skin barrier.

Without the need of frequent dressing changes and sustainable fashion over a longer period, controlled drugs delivery system (DDS) can provide an excellent delivering of drugs to the wound sites, reducing the patient exposure to an excess of drugs beyond the dosage which is required for wound healing, and even skin tissue engineering. Drugs delivery systems are potentially useful in the treatment of local infections where it might be beneficial to avoid high systemic doses while increasing local delivery concentration of antibiotics, it is possible to release active substances to the wound sites in a controlled way for sustained period, about a week or more, it could be made by bioadhesive, naturally, synthetic and semi-synthetic derived from polymeric dressing (Lee & Park, 2000).

When dealing with patient compliance, DDS have advantages, especially in the treatment of chronic wound management, where most of the patients undergo for a long treatment period. In addition to that, these systems are biodegradable and therefore can be easily washed off the wound surface, after they exerted their proposed effect (Rao & Devi, 1988).

Nowadays, most of the modern wound dressing are made from polymers which can play a role in the release and delivery drugs to the wound sites. Biodegradable polymeric microencapsulate dressings have received great attention as a potential drug delivery vehicle in consideration of their applications in target drug delivery, once that they are employed for controlled drug delivery to wounds include. The modern wound dressing can be classified as hydrogel dressing, hydrocolloid dressing, semi-permeable adhesive film dressing, biological dressing and foam dressing (Burrell & Morris, 1998; Ishihara et. al., 2006; Katti et. al., 2004; Maeda et. al., 2001).

In the 21st century, drugs microencapsulation technology has been applied for drugs delivery. It possesses a significant potential for therapeutic and pharmaceutical fields, as it provides controlled and sustained release of pharmaceutical agents for various medical proposes. It is necessary to have some technical consideration regarding the futures and requirements of microencapsulated pharmaceutical agents; these agents must be identified and specified before the drug design. Ethical approval of pharmaceutical products should be obtained to demonstrate the use of the drugs, as being supported by results of clinical trials and animal's experiments. Lucas et. al. (Naves et. al., 2016), mentioned that according to the EU 7th amendment of the Cosmetic Directive, the studies *in vivo* should only be performed when it is not possible to achieve reliable and better scientific outputs *in vitro*, therefore, *in vivo* studies should be replaced for *in vitro* models and procedures. Economic, quality and the technical issue should be considered when the pharmaceutical agents microencapsulated technology is employed for the mass

production. When these drugs are designed for the therapeutic application, is very important to identify and examine carefully the chemical, physical and therapeutic properties of microcapsules (Naves et al., 2016). To avoid an overdose administration of a drug, the drug entrapment amount in microcapsules should be cautiously controlled. In addition to that, the particle size of microcapsules designed could alter the total surface area for the drug release. The quality control is necessary for producing microencapsulated medicine with an acceptable quality (Orive et. al., 2004).

Microencapsulation technology may provide many benefits in transdermal drug delivery. Microencapsulation provides a physical barrier to protect the drugs which are liable to external environments such as evaporation, heat, alkalinity, acidity, oxidation or moisture before releasing the drugs in order to improve its stability. It also improves the drawbacks of conventional drugs delivery routes, including poor compatibility and bioavailability, short life and absorption. When thought in a specific therapeutic purpose the long-term therapeutic performance could be enhanced by microencapsulating drugs with an appropriate host response, the drugs can be released at once or moderately and gradually (Bansode et. al., 2010). All the materials employed on the development of the microcapsules formation should be standardized in terms of purification, reaction situations and chemical compositions to achieve the efficiency and biosafety requirements and minimize the variability production in different laboratories (Orive et. al.2003).

In 2013, Lam and Gambari stated (Lam & Gambari, 2013):

> *"Transdermal delivery is to apply the medicine drug to the skin, the drawbacks of oral administration such as enzymatic degradation and rapid clearance in the gastrointestinal tracts or the first pass metabolism therefore can be avoided. Drug delivery target the skin sited alleviates the physical pain and discomfort and promotes the patient convenience and compliance for drugs treatment. Although the skin offers a relatively large and readily accessible surface area for drugs absorption, the skin target drugs delivery still present limitation. The human skin is an impermeable barrier that provides a strong protection against external substances... Skin is mainly composed of two layers: the underlying layer and upper basement layer. In the underlying layer, various types of cells, blood vessels, lymphatics, and nerves are found as a dense network of connective tissue. In the upper basement membrane, more than 90% of stratified keratinocytes exist, and keratinocytes undergo the cell differentiation and move upwards from the stratum basale, through the*

> *stratum spinosum and stratum granulosum, to the outermost layer, the stratum corneum to finally become corneocytes... The stratum corneum, as an outermost layer of the epidermis, provides an extremely effective barrier for the control of drug penetration by an incapability of large majority of drugs to pass the skin at therapeutic rates whereas it is also the main barrier to spread the water out of the skin... Therefore, the major challenges of transdermal drugs delivery are to overcome the strong barrier function of the skin as this barrier lead to slow drugs penetration rates, limited drugs uptake rate, lack of dosage flexibility or precision."*

Drugs topical delivery is related to the application of drugs to the skin surface, aiming to delivery therapeutic agents through skin into the systemic circulation or bloodstream (transdermal delivery) or to pathological sites of the skin (dermal delivery) (Garvie, 2016). As an example of skin disorder treated using transdermal delivery we can mention severe pain which is treated with fentanyl (Delgado & Guy, 2014), while eczema and psoriasis are treated using dermal delivery (Flowers, 2004). The efficacy of topical drugs delivery is directly related to the diffusion of the therapeutic agent through the skin and the drug formulation.

When compared to intravenous and oral delivery, the administration of drugs by dermal and transdermal routes are advantageous in several aspects. Once that the formulation is applied directly to the affected site of the skin, it is possible to minimize or even eliminate any adverse side effect which may occur for the oral treatment of skin disease, as a lower dose is needed. Oral treatment requires higher systemic concentration of the drugs to achieve the therapeutic effect at the disease site. The concentration of the dose, once that passes through the metabolism, in case of oral administration, involves a peal of drug concentration in tissue, organs, and blood, this is followed by a decline. Transdermal or dermal delivery, allow lower daily dose.

In addition to that, it can maintain the drug level with the therapeutic windows for prolonged time, reducing the frequency of dosing required and extending the duration of the action of the drug. When using a patch or scaffold as we propose in this study, the input can be determinate simply by its removal (Garvie, 2016). In **Figure 2-16** we can obverse several biomedical applications of nanofibers composites.

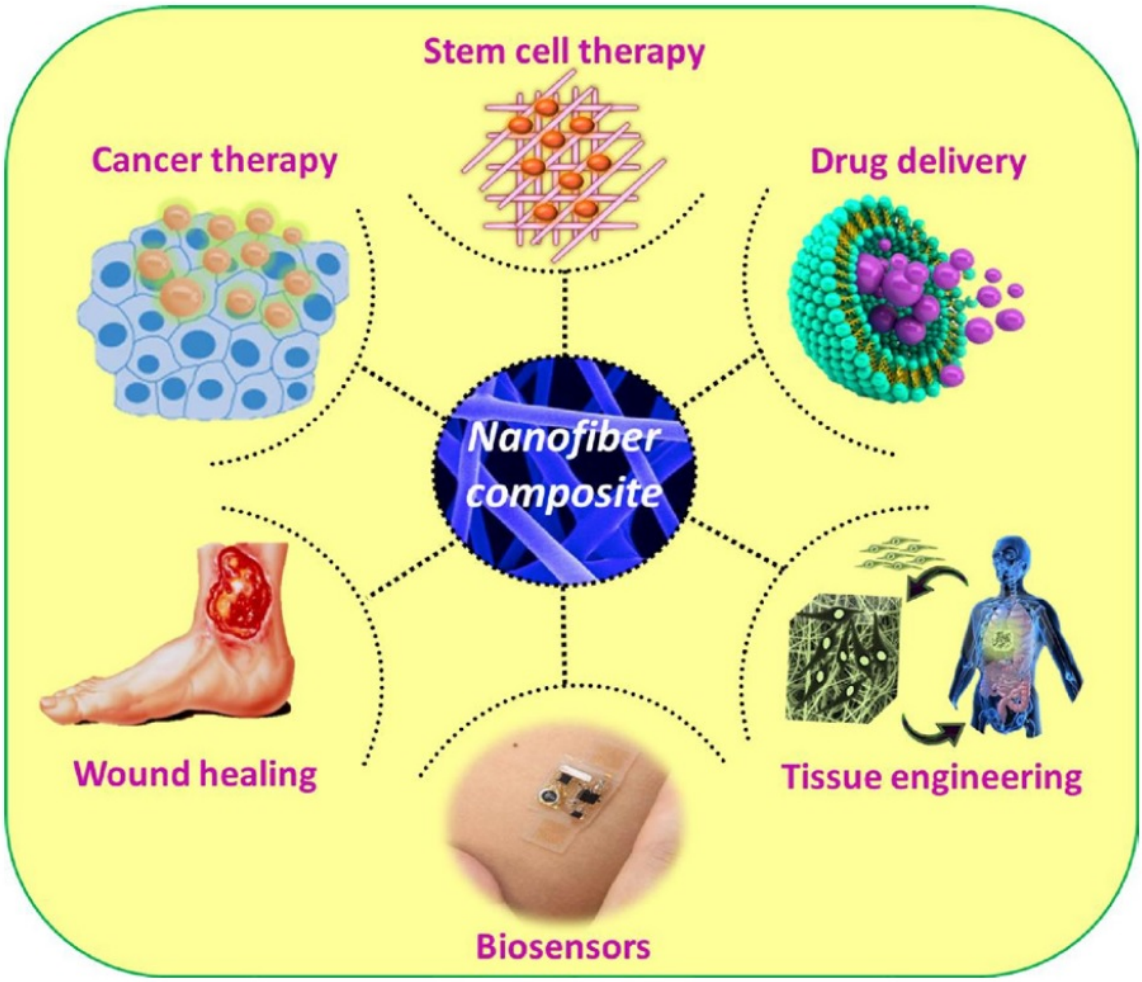

Figure 2-16 Biological application of nanofibers composites. Source: (Ramalingam & Ramakrishna, 2017), p11.

2.3.2 Permeation Pathways

The drugs penetration rate can vary from one skin type to the other, depending directly upon the application site, the type of skin disease under treatment, age, race, etc. An ideally transdermal DDS should allow a considerable amount of drugs to overcome the skin barrier, providing adequate release of the drug formulation. The patient's health condition can be worsening if the development of the drug is not biocompatible, leading to skin irritation and cytotoxicity of healthy cells. Some researchers have reported the enhanced cell proliferation of human dermal fibroblast (HDF), by using biocompatible polymers on formulation and development of nanofibrous wound dressing for skin tissue engineering (Jin et al., 2013).

The permeation of drugs through (trans) dermal route is possible to be achieved by three main pathways, in which the drugs can penetrate the stratum corneum, these routes are namely: intercellular domains

(intracellular), cells themselves (transcellular) and via appendages (appendageal) **Figure 2-17**. The percutaneous absorption of active compounds topically applied is directly related to the density of appendages such as sweat glands and hair follicles **Figure 2-18**, nature of permeating molecules. Depending upon the route of drugs delivery is possible to delivery either to the skin surface or into systemic blood circulation, at the site of application.

The intercellular is believed to be made by uncharged molecules, in which the major pathway for the permeation is small (< 500g mol^{-1}) (Bos & Meinardi, 2000). The compound permeation in this route occurs by diffusion across lipid lamellae. Providing the limiting step rate, which these intercellular lipids contribute to the continuous phase within the stratum corneum, from lamellar structures between the corneocytes.

The transcellular is a route that needs repeated penetration of the permeating compounds into and out the intercellular lipids and the corneocytes. In this route, the compounds at the SC must diffuse through both hydrophilic regions, which is hydrated keratin within corneocytes and the lipophilic regions, the lipids (Garvie, 2016)

The appendages can penetrate the epidermis and the stratum corneum, which can provide a route known as bypass barrier. This route can vary the density of the appendages, consistently low related to the anatomical site. This low resistance route can play an important role in ionophores, important for low SC diffusivity, such as a large molecular weight of active and hydrophilic compounds.

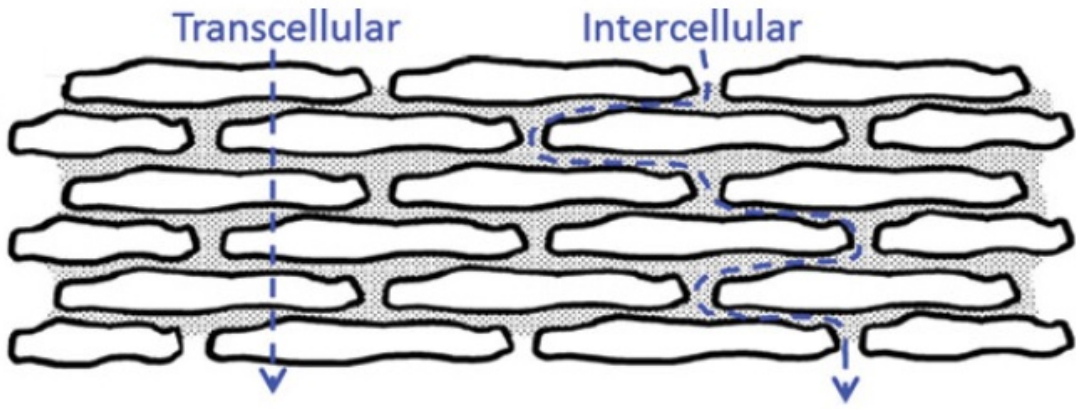

Figure 2-17 Percutaneous absorption of drugs topical application, namely transcellular and intercellular routes. Source: (Garvie, p6, 2016).

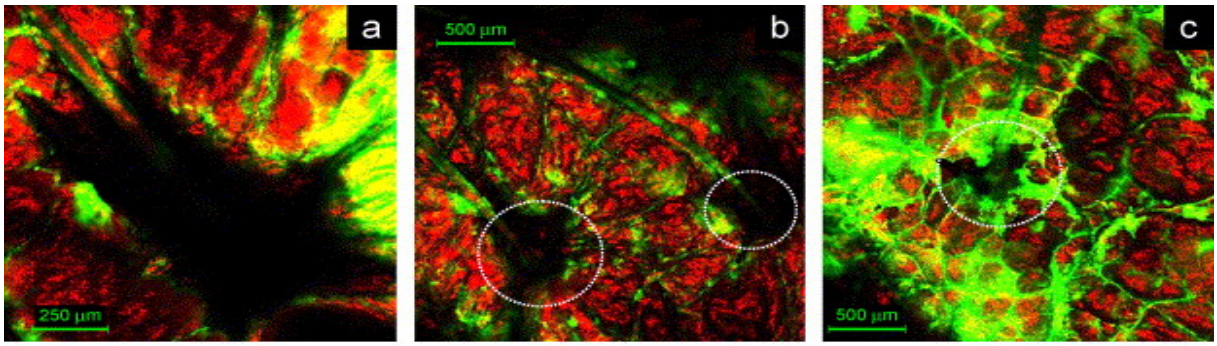

Figure 2-18 Images showing skin surface and saturated absorption of isothiocyanate followed by 30 min (a), 1 h (b), and 2 h (c). Possible nanoparticle route through a follicular pathway. (Alvarez et. al., 2004). With permission from Elsevier.

2.3.3 Implications of Nanofibers Based in DDS in Healthcare

Extracellular matrix (ECM) based on nanofibers, imitated their surface functionalization capabilities and the inter-connected fibrous morphology, so for these reasons the composite nanofibers can offer a potential platform to immobilize/incorporate therapeutic agents to develop and provide local drugs delivery systems (DDS), owing to their nanoporosity and high surface area (Hu et al., 2014). Acting as efficient carriers for the pharmaceuticals, genes and growth factors, the nanofibers-based DDS ensure its application in various medical fields including tissue engineering, advanced pharmaceuticals, surgical implants, cancer therapeutics, wound dressings, etc. (Kanani & Bahrami, 2010). Synthetic and natural polymers for scaffold design can be employed for drug functionalization, encapsulation or crosslink strategies. The polymers applied are directly dependent upon the therapeutic requirement such as the mode of drug release (biphasic/triphasic/pulsatile/immediate) and drug release kinetics (delayed/burst/slow). **Figure 2-19**, allows us to see different nanofiber composite design explored as DDS, thus electrospinning technique a variety of nanocomposites morphologies and structural flexibility can be fabricated, e.g., layered nanocomposites, hybrid composites and core-shell nanocomposites structures, which will later control the drug release profile of the proposed therapeutic agent.

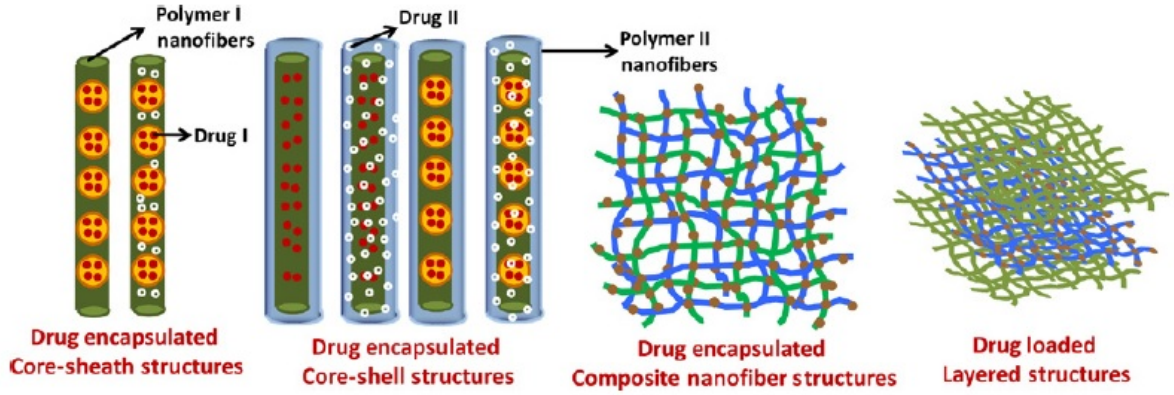

Figure 2-19 Composite nanostructures used as DDS. Source: (Dhand et. al. 2017, p.201).

For wound dressing application, with their ability to mimic the functional and structural biology of ECM, nanofibrous meshes may help to promote the hemostasis of injured tissue, allow cell respiration (due to high porosity) and absorbing wound exudate thanks to its high surface area. In addition to that, the nano dimension pores size, they prevent infiltration of external microorganism at the wound area, in this manner it can discourage cell/tissue infiltration. Active wound dressing nanofibers can be designed by incorporating growth factors, antimicrobial ingredients (*Aloe vera,* honey), antimicrobial metals ions (Cd^{2+}, Ag^{+}, Cu^{2+}, etc.). For tissue engineering applications, the nanofibrous scaffolds provide ECM- mimetic physical support which is very similar to the fibrous proteins of native ECM, leading to better regeneration and growth of the tissue. It is possible to open new avenues for designing organ remodeling, site-specific DDS, and artificial organ implants by incorporating therapeutic genes, antibiotics, growth factors, etc. All these properties together provide nanofibrous scaffolds many benefits towards healthcare application and use (Abrogo, McArthur, & Kingshott, 2014; Rujitanaroj, Pimpha, & Supaphol, 2008).

2.3.4 Wound Healing

Wound healing is a dynamic and complex process by which the body tissue or even the skin repairs itself after injury. When dealing with chronic wounds there is a need for the development of new wound healing products, focusing proper functioning of human organ system and faster healing of wounds. Before choosing a wound dressing some factor should be considered, as the size of the wound, volume of exudate, health of the patient, type of the wound, the presence of infection, physicochemical properties of the wound dressing (Dhand et. al., 2017). Electrospun mats have shown among many options great promise for formulating wound healing products, known as a wound dressing, it is pharmaceutical compress or sterile pad which is applied in contact to the wound to stimulate or assist the healing process

and prevent the wound from any form of infection. The mats can be used as wound dressing to accelerate the wound healing process, however, if not managed effectively in timely manner and application the wound can directly interfere with the patient wellbeing.

There are some characteristics ideal for wound healing which include absorption of wound exudates, bacterial barrier, moisture maintenance, ease of removal, hemostatic efficiency, space for gas exchange and low cost. To prevent microbial infection at the wound site, one of the most common approaches includes the blending of polymers with antibacterial nanoparticles, this may act synergistically (Ebrahimi et al., 2016). Researchers have been investigating the various combination of biocompatible electrospun mats with different nanoparticles grafted for speeding up the healing process. For instance, Ebrahimi-Hosseinzadeh and colleagues reported the *in vivo* results showing the efficacy comparison of the commercially available ChitoHeal Gel and gelatin/hyaluronic acid (GE/HA) electrospun mats with diameter range size of 20 to 150 nm (Ebrahimi et al., 2016). The results, see **Figure 2-20**, of this study, showed an improvement of wound closure percentage for ChitoHeal gels and GE/HA nanofiber composite of 77.8% and 81.9% respectively, untreated control group show 65% of wound closure. It was demonstrated that the dermal defect recovered back to its normal condition within 14 days postoperation. The histological results confirmed lesser inflammatory cells and increased epidermis production in ChitoHeal gel and GE/HA treated group compared to the control.

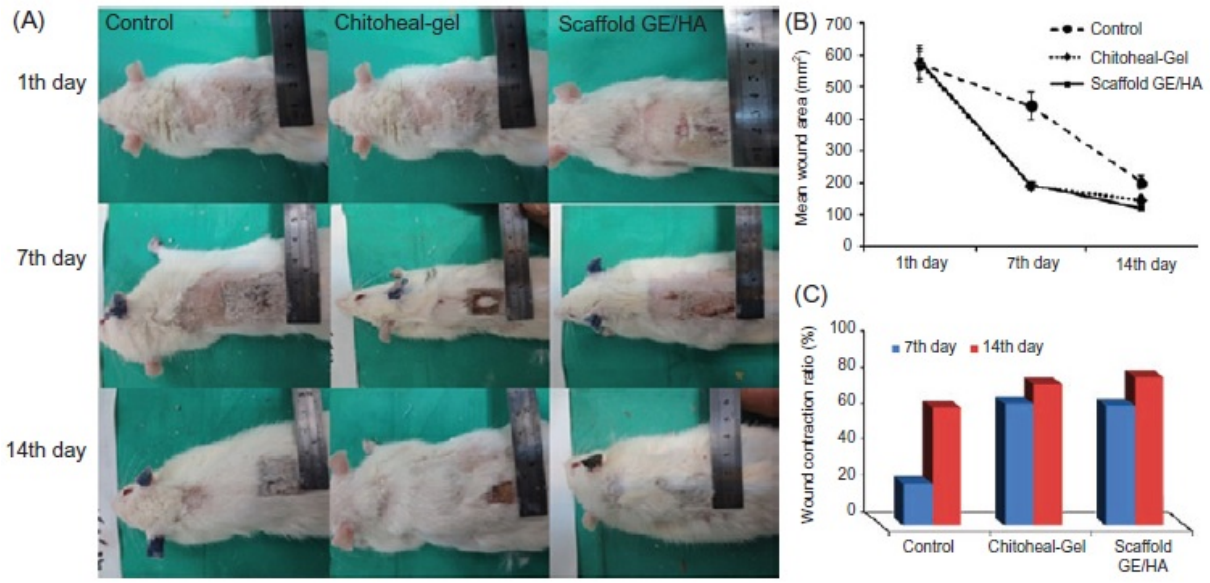

Figure 2-20 (A) Macroscopic appearance of skin wounds treated with gauze as control, ChitoHeal gel (containing chitosan) and GE/HA nanofibrous at day 1, 7 and 14 in excision wound model. (B) The assessment of burn wound healing. (C) Wound contraction ratio of the wound treated with control, ChitoHeal gel and GE/HA nanofibrous membrane. Source: adapted from (Ebrahimi et al., 2016).

2.3.5 Cancer Therapy

Cancer is one of the deadliest diseases, showing continuously increased incidence and mortality rates worldwide (Chen et al., 2015). Cancer treatment remains a challenging task, especially when dealing with prevention or completely eradication of cancer cells without damaging the rest of the body. It is well known that for cancer treatment are commonly used traditional therapies such as chemotherapy or radiotherapy. Chemotherapy is the most engaged treatment, using anticancer drugs, which often fails because of the highly toxic effects of the chemotherapeutic drugs, reported to have enormous side effects to the patients. The main cause for the failure to account for the discrepancies encompassing cancer therapies are inadequacies in the administration of therapeutic agents, with uncontrollable and lack of selective features. In the last few decades, researchers are focusing their research, to minimize the side effect in the other body sites of the patient and increase the therapeutic effect to the carcinogenic target tissue. These efforts are thanks to the development of smart drugs delivery systems, possibly due to the development of nanotechnology, exploring potential new nanomaterials, including polymeric micelles, polymeric nanoparticles, DDS for cancer therapeutics, dendrimers structures, liposomes, carbon nanostructures (CNT, fullerene), etc. Due to dedicated and consistent research efforts, many of these approaches have been approved for clinical trial and are nowadays commercialized in the market (Dhand et. al., 2017).

Recently, drug-loaded composite nanofibers have attracted enormous attention from the scientific community for use in controlled and local drug delivery in cancer therapy. Due to technological advances, nanofibers composites are emerging as an alternative strategy for cancer prevention and treatment. They possess the ability to mediate drug delivery with prolonged exposure of the drug to the carcinogenic cells, improving the targeting ability of the drugs, as well the solubility of water-insoluble drugs (Munaweera, 2015). Chen and co-workers (Chen et al., 2015) developed some multifunctional electrospinning composite fibers for orthotopic cancer treatment *in vivo*, which they stated:

> *"DOX-loaded $NaGdF_4$: Yb/Er@$NaGdF_4$: Yb@mSiO_2-polyethylene glycol nanoparticles were incorporated into PCL and gelatin loaded with antiphlogistic drug MC, to form MC/UCNPS/DOX CFs by electrospinning. The resultant multifunctional spinning pieces can be surgically implanted directly at the tumour site of mice as part of orthotopic chemotherapy by controlled-release DOX from mesoporous SiO_2 and the upconversion fluorescence/magnetic resonance dual-model imaging through $NaGdF_4$:Yb/*

> *$Er@NaGdF_4$:Yb embedded in MC/UCNPS/DOX in vivo. Additionally, MC in MC/ UCNPS/DOX CFs suppressed the inflammatory response, which helped in wound healing in vivo. These results provide an encouraging prospect of using drug-loaded electrospun nanofibers in orthotopic diagnosis and treatment combined with current treatment protocols, especially for those patients suffering from unresectable tumours or metastases cancer."*
>
> (Chen et al., 2015)

In **Table 2-4**, we show some composite nanofiber system explored as DDS for cancer therapeutics. Drug-loaded composite electrospun mats can release controlled drug by properly designed architecture, mode of drug incorporation, fiber diameter, the composition of nanofibers and porosity (Rujitanaroj et al., 2008).

Table 2-4 Drugs delivery system based on electrospun nanofibers reported for cancer therapeutics.

Polymer	Additive/filler	Anticancer drug	Reference
PCL,Gelatin	Unconventional core/shell Silica NPs	Doxorubicin, indomethacin	(Hou et al., 2013)
PLLA	Titanium in the form of Titanocene drug	Titanocene	(Ping Chen et al., 2010)
Self- assembled Peptide	Platinum drug	Cisplatin; *Cis*-dichlorodiammine platinum	(J. Kim, Anderson, Jun, Repka, & Jo, 2009)
PLGA	CNT	Doxorubicin	(Yu, Kong, Li, Li, & Yan, 2015)
PLLA	CNT	Doxorubicin	(Z. Zhang et al., 2015)
PLA	Platinum drug	Cisplatin	(P. Chen, Wu, Ding, & Zhu, 2011)
PLLA	Fullerene/C_{70}	Paclitaxel	(Liu, Wei, & Chen, 2014)

Abbreviations: PCL – polycaprolactone, PLLA - poly-L- lactic acid, PLGA - poly(lactic-co-glycolic acid), CNT - carbon nanotubes, PLA - poly-lactic acid.

2.4 Biofunctionalization of Fibrous Scaffolds

For the use of therapeutic and advanced biological application, it is essential that the surface of the electrospun mats are either incorporated or coated with bioactive molecules or bioactive agents. During the fabrication of tissue scaffolds, certain natural or synthetic polymers can be used, once they may have unique biological functions. Coating natural polymers on the surface of synthetic polymers fiber is one approach which can be used for biofunctionalization. As another alternative, it is possible to mention,

aiming to obtain drug release of fibrous scaffolds, the hydrophobic therapeutic drugs that can be directly blended into the electrospinning polymer solution (Baji & Mai, 2017).

2.4.1 Interactive Characterization of Cells to Nanofibers

When using biomedical application by developing nanofibers, it is crucial to determine the interaction cell to cell, and cell to the scaffold. This aims to evaluate the cell adhesion to the electrospun mats, the proliferation, tissue regeneration and the most important features which are the drug delivery profile and the cytocompatibility, that will be better explained in the following sections (Pasricha & Sachdev, 2017).

2.4.2 Cytocompatibility

Over the last few years, we have observed the increase of use of nanofibers in the field of pharmaceutical and biomedical studies. Thus, it has become significantly important their potential cytocompatibility, and the characterization of their effects after exposure to the biological system. Comprehensive study and detailed understanding of chemical-physics and nano-bio interactions of nanofibers are important to prevent the risk of materials failure at preclinical or late clinical stages. Studies such *in vitro* and *in vivo* are important assays to characterize the cytocompatibility of all electrospun nanofibers, either natural or synthetic polymers-based. These studies are important for skin, tissue and cell engineering applications. They also indicate that the cellular response depends on cell type, nature of scaffold and surrounding medium (Yoshimoto, Shin, Terai, & Vacanti, 2003).

In vitro cytotoxicity has become an integral aspect of drugs discovery because it is cost-effective, predictive and convenient means of characterization the toxic potential of new chemicals entities. The observation of toxicity testing is critical and a necessary research and industry practice to define and identify the safety thresholds for new potential drugs for drugs delivery including chemotherapeutics. Niles and co-colleagues state that:

"The advent of in vitro cytotoxicity testing has greatly streamlined this process and is now considered to be a nearly compulsory activity starting at target validation and continuing through medicinal modification. Unlike animal-based toxicology testing, there are clearer definitions and greater agreement for what constitutes cytotoxicity in vitro." (Niles, Moravec, & Riss, 2009)

The cytotoxicity refers to a treatment or compound if it can prevent cellular attachment, leading to a reduction in overall viability, adversely affects replication rate or cause dramatic morphological changes.

It is important to note that the manifestation of these effects are directly related to the mechanism of cytotoxicity and length of exposure (Riss & Moravec, 2004).

Both *in vitro* and *in vivo*, give us the important characterization of nanofiber, the cells response and sense to the physical properties of the electrospun mats matrix by covering mechanical cues into intracellular chemical signals, which in turn control phenotypic behavior, protein production and gene expression (Pasricha & Sachdev, 2017).

2.4.3 Cytotoxic Assays

The basis to understand and have a better insight regarding the reaction of the potential biomaterial when present in the body is through cell culture methods. Cells cytotoxicity assay, chemistries are formulated to detect loss of membrane integrity associated with cell death (Niles et al., 2009). It is called *in vitro* testing methods. In this type of testing model, there is a selection of a specific cell lineage to mimic a response likely sensitized or observed by biomaterials, scaffolds or particles *in vitro.* In 2008, Williams suggested:

> "*The biocompatibility of a long-term implantable medical device refers to the ability of the device to perform its intended function, with the desired degree of incorporation in the host, without eliciting any undesirable local or systemic effect in that host... The biocompatibility of a scaffold or matrix for tissue engineering products refers to the ability to perform as a substrate that will support the appropriate cellular activity including the facilitation of molecular and mechanical signaling systems, in order to optimize tissue regeneration, without eliciting any undesirable local or systemic responses in the eventual host.*"
>
> (Williams, 2008)

Currently, the most used cytotoxicity biomarkers are conserved, constitutive and relatively stable, high-abundance enzymes "released" into the extracellular environment following the loss of membrane integrity. This extra-cellular environment is also called as culture medium. The detection of cytotoxic biomarker activities outside the membrane compartment after treatment is referred as proof-positive for compound´s cytotoxic effect. On the contrary, the viability assays, the decline in cell response is interpreted to be caused by cell death or cell-cycle arrest. It is important to note that enzymatic decay as a function of time in the culture medium can lead to underestimation of the absolute level of cytotoxicity.

Activity-based surrogates of cell death, can have their utility limited, due to a finite enzymatic half-life during long compound/cell contact incubation (48-72 hr) (Niles et al., 2009). When performing *in vitro* tests, it is crucial to observe if there is no false positive or false negative outputs from the study. This may happen due to the fact that most of the cells cultures are very sensitive to changes in their environment and experimental condition, such as, necessity of proper UV sterilization of the fume hood which may lead to cross-contamination, pH of the medium, lack of antibiotics into the medium, chamber temperature and O_2, incubation time, medium changes, following the protocols (Goonoo, Bhaw & Jhurry, 2014a). Therefore, it is crucial to control the experimental conditions to ensure that the measured cells death does not correspond to the toxicity of the added electrospun mats but instead as a result of unstable culturing conditions (Pasricha & Sachdev, 2017).

BSI, ASTM, and ISO are some standard protocols used for cell culture assays, to evaluate the biocompatibility. These protocols are morphological assays, wherein the outcome provided is measured by observation of any changes in cell morphology (Jones & Grainger, 2009).

When performing cytotoxicity assay, each biomarker has its own advantages and disadvantages. As we can observe in **table 2-5**, luminescent formats tend to delivery maximal signal windows in a rapid but prolonged way, offering additional utility over fluorescent format method regarding sensitivity detection and cost associated, whereas fluorescent format requires short incubation period with the sample (Niles et al., 2009)

Table 2-5 Viability and cytotoxicity Assay Reagents for High Through Screening (HTS). Source: Adapted from (Niles et al., 2009).

Assay Type	Reagent Name	Reagent Incubation	Biomaker	Detection Method
Cytotoxicity	CytoTox-Glo™	15 min	Protease(s)	Luminescence
	CytoTox-Fluor™	30 min	Protease(s)	Fluorescence
	CytoTox-ONE™	10 min	LDH	Luminescence
Viability	CellTilter Blue®	2 – 4 hs	Reductase(s)	Fluorescence
	CellTilter Fluor™	20 min	Protease(s)	Fluorescence
	CellTilter Glo®	10 min	ATP	Luminescence
Cytotoxicity and Viability	MultiTox-Fluor	30 min	Protease(s)	Luminescence/ Fluorescence
	MultiTox-Glo	15 – 30 min	Protease(s)	Luminescence/ Fluorescence

2.4.4 Activity test of cells metabolisms

The exposure to certain cytotoxic agents can compromise the cell membrane, allowing the cellular contents to leak out. Quantitative viability test based on this includes trypan blue and neutral red assay (Lee et. al.2006). A weak cationic dye, toluylene red or neutral red, can cross the plasma membrane by diffusion, tending to accumulate into the lysosomes within the cell. The discernment between live and dead cells is possible if the cell membrane is altered, then the uptake of neutral red is decreased and can leak out. The cytotoxicity can be quantified by taking spectrophotometric measurements of neutral red uptake at 540nm. The output intensity of the color obtained is inversely proportional to cytotoxicity of scaffolds and proportional to the viability of the cell population (Goonoo, Bhaw & Jhurry, 2014).

Another commonly used colorimetric assay is alamar or resazurin blue, where the non-fluorescent alamar blue dye is reduced to a pink fluorescent dye by cell metabolic activity mainly by acting as an electron acceptor for enzymes such as NADP[7] during oxygen consumption (Goonoo et al., 2014a).

A different approach is the lactate dehydrogenase release monitoring, known as LDH assay. In this assay the LDH is released from damaged cells, then it oxides lactate to pyruvate, promoting conversion of tetrazolium salt INT to formazan, which is a water-soluble molecule with the absorbance of 490 nm. The LDH released in this assay is directly proportional to the number of lysed or damaged cells (Haslam, Wyatt, & Kitos, 2000).

2.4.5 MTT and MTS Assays

The most used tests for viability are MTT and MTS:

- **MTS assay** (3-(4,5-*dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium. "This assay consists of a cell-permeable, soluble tetrazolium salt, and electron coupling reagent (phenazine methosulfate, PMS) that is added to the cellular environment after the appropriate dosing periods. During incubation with MTS, the dehydrogenase enzymes present within the mitochondria of healthy cells reduce the MTS into a dark purple, water- soluble formazan compound, which can be quantified with a microplate reader at 490nm. Absorbance values measured at zero incubation time are subtracted from the final readings to account for*

[7] Nicotinamide adenine dinucleotide phosphate, abbreviated NADP. provides the reducing equivalents for biosynthetic reactions and the oxidation-reduction involved in protecting against the toxicity of reactive oxygen species (ROS).

background absorbance due to the NPs or the solution. The changes are compared to controls ... and percentages are calculated" (Schrand, Lin, & Hussain, 2012).

- **MTT assay***: "The biochemical mechanism behind the MTT assay involves NAD(P)H-dependent cellular oxidoreductase enzyme that converts the yellow tetrazolium MTT [3-(4, 5- dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide] into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)- 1,3-diphenylformazan (formazan). The formed formazan can be dissolved with dimethyl sulfoxide (DMSO) to give a purple color with characteristic absorption at 540 nm. The Intensity of purple color is directly proportional to the cell number and thus indicating the cell viability"* (Schrand et al., 2012).

2.4.6 Cells studied

2.4.6.1 Human Dermal Fibroblast Cells

It is known that human dermal fibroblast (HDF) and extracellular matrix play a pivotal role during wound healing process. When the skin is injured, dermal fibroblasts (dFb) and putative precursor cells from adjacent intact dermis are attracted to the fibrin-rich wound bed where dFb differentiate and proliferate to myofibroblasts (MFb) upon stimulation by local cytokines (Watarai et al., 2015). Transforming growth factor beta 1 (TGFβ) and platelet-derived growth factor (PDGF) and macrophages are functional mediators supporting differentiation and proliferation (Werner & Grose, 2003). This process is followed by the differentiation from dFb to alpha smooth muscle actin (αSMA) expressing MFb in the presence of mechanical stress and TGFβ. Finally, the differentiated MFb can synthesize major components of the granulation tissue like fibronectin and collagen fibers, contracting the wound (Tomasek et. al., 2002).

In case of disturbed wound healing such as chronic venous insufficiency, diabetes or infections, and large wounds as burns, engineered biomaterials should provide an effective sterile and fast wound closure, at the same time facilitate the initiation of endogenous wound repair. In that context biomaterials should be tested in dFb, to assure its effectiveness by forming a beneficial micro-milieu that provides key functions of the natural extra-cellular environment, supporting differentiation, adhesion, and proliferation of dFb (Watarai et al., 2015).

2.4.6.2 Murine Melanoma Cells (B16)

Malignant melanoma cells have enhanced proliferation and survival abilities, the major reason for this behavior is their anti-apoptosis capacity, which is a problem faced by clinical chemotherapy drug tolerance (Zhang et al., 2014). In addition to that, chemotherapy treatment is very invasive for patients having a low self-immune defense. This is a complex disease that arises through multiple etiologic pathways. A limited number of treatments are available for melanoma medical application, most of patients with a more aggressive form of the disease with neither long-lasting nor effective treatment presently exists decline treatment (Yang et. al., 2013). Although, the major etiologic agent in the development of skin cancer is ultraviolet radiation (UVR) exposure, which is responsible for some gene mutation, such as MAPK- ERK, which includes the cascade of BRAF, NRAS, MEK1/2 and ERK 1/2proteins, involved in the control of cell proliferation, growth and migration (Colombino et al., 2012). Mutations in this pathway may play a major role in the progression and development of melanoma cancer (Casula et al., 2009). The National Comprehensive Cancer Network (NCCN) guidelines metastatic melanoma can be treated with dacarbazine (DTIC) which is FDA drug approved (Jilaveanu, Aziz, & Kluger, 2009), also with paclitaxel, temozolomide; these treatments are not specific MEK and BRAF inhibitors (Anderson et al., 2013).

3 CHAPTER

EXPERIMENTAL DEVELOPMENT

The experimental session of this doctoral thesis was possible, due to the agreement between the Centre for Textile Science and Technology, University of Minho- Portugal, the National University of Singapore - NUS (Center for Nanofibers & Nanotechnology - Department of Mechanical Engineering), Singapore Eye Research Institute - SERI (Department of anti- infectiveness research) and Nanyang Technological University- NTU – Lee Kong Chian School of Medicine (Department of experimental medicine).

As a multidisciplinary research, it was necessary to follow the specific and unique guidelines of each research group. Different steps of the practical part were made in different institutes, so the protocol used to develop each of the following topics are described as used at the department. The protocol may differ from one University to the other. We followed all the chemical and biological safety procedures, accordingly to in-house training of each department.

For this study we used Linear (Mn~20,000, catalogue no. 764965, Sigma) and branched (Mn ~10,000, catalogue no. 408727, Sigma) polyethyleneimine hydrochloride, Polycaprolactone (Mn~80,000, catalogue no.440744, Sigma), Dulbecco's Modified Eagle's Medium (DMEM), antibiotics, trypsin-EDTA and fetal bovine serum (FBS) were purchased from Gibco (ThermoFisher Scientific, Singapore). CellTiter 96® AQueous One solution was purchased from Promega (Singapore). Human dermal fibroblast and melanoma B16 culture was from American Type Culture Collection (Manassas, VA, USA).

3.1 Electrospun Mats

For this study were developed 5 different mats, 10% w/v of polycaprolactone (PCL), 10% w/v of PCL blended with 2% and 5% of linear polyethyleneimine (LPEI) and 10% w/v of PCL blended with 2% and 5% of branched polyethyleneimine (BPEI), respectively. All the mats were based on 10% w/v of PCL, which was prepared from methanol and chloroform (3:7) solution, left overnight steering at 200rpm, room temperature. Identical conditions were used for the preparation of mats containing 2% and 5% of LPEI

mats and 2% and 5% of BPEI mats. The solutions left overnight were transferred to a polypropylene plastic syringe with 27G stainless steel blunted. The solution of each copolymer was extruded at an applied voltage of 15kV from a high voltage power supply (Gamma High Voltage Research, Inc., FL, USA), and the distance between the collector and the needle (flattened aluminum foil with cover slips (15mm) was set at 13 cm at a feed rate of 1ml/h (KD 100 Scientific Inc., MA, USA). The electrospinning process was made for 45 minutes for all mats. For simplicity, the mats were labeled as follows: PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI. The development process of electrospun mats as well the labeling and sterilization methods can be observed in **Figure 3-1, Figure 3-2** and **Figure 3-3**, respectively.

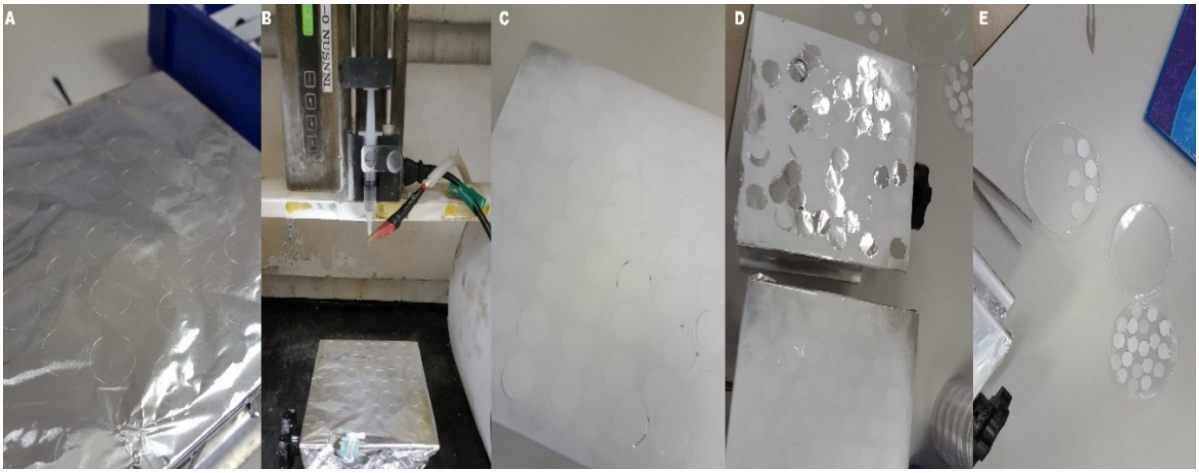

Figure 3-1 Electrospinning process. (A) coverslips on aluminum foil. (B) Electrospinning apparatus. (C) Nanofibers on coverslips. (D) Removing electrospun mats from aluminum foil. (E) Separation of different of electrospun mats for further tests.

Figure 3-2 Labeling the electrospun mats for biological tests. of PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI. Abbreviations: PCL: -poly-(ε-caprolactone), PCL_2LPEI: poly-(ε-caprolactone) blended with 2% of linear Polyethyleneimine, PCL_5LPEI: poly-(ε-caprolactone) blended with 5% of linear polyethyleneimine, PCL_2BPEI: poly- (ε-caprolactone) blended with 2% of branched Polyethyleneimine, PCL_5BPEI: poly-(ε-caprolactone) blended with 5% of branched Polyethyleneimine, TCP: tissue culture plate.

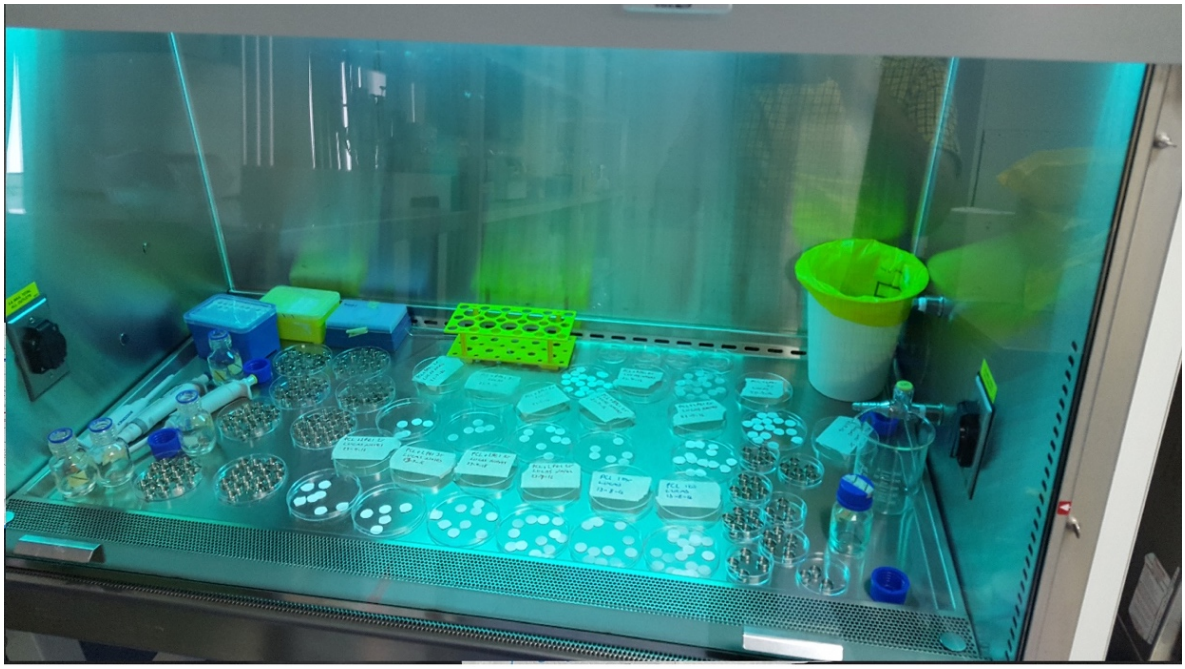

Figure 3-3 Sterilization of all electrospun mats prior biological test, under UV radiation for 30 min in the fume hood.

3.2 Water Contact Angle (WCA)

The surface wettability of electrospun mats was determined by dynamic water contact angle measurements on a VCA Optima Surface Analysis system (AST products, MA, USA). This assay was carried by dropping 1µl of distilled water on the surface of the mats. The reported values were determined from three independent triplicate experiments, after 8 seconds of plasma radiation for hydrophobicity surface modification. This procedure was performed for all mats. Water contact angle experiment can be observed in **Figure 3-4** and **Figure 3-5**.

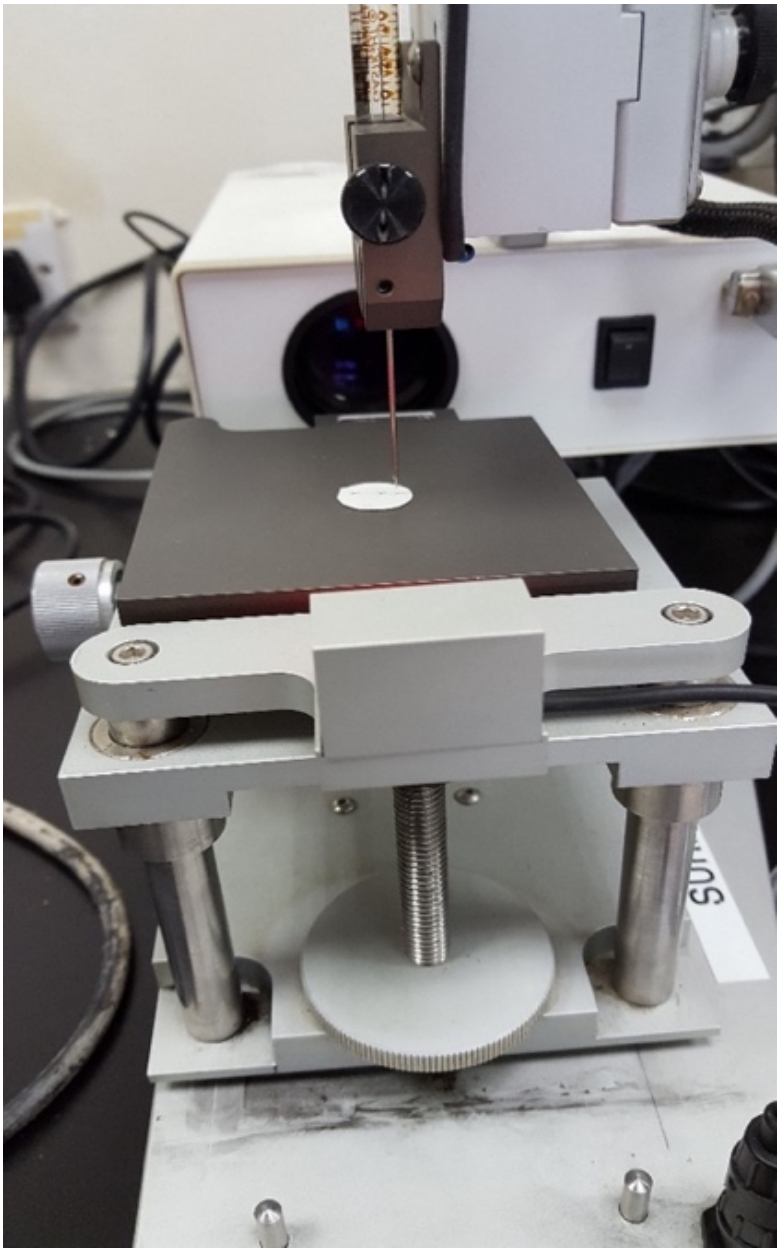

Figure 3-4 Water contact angle apparatus.

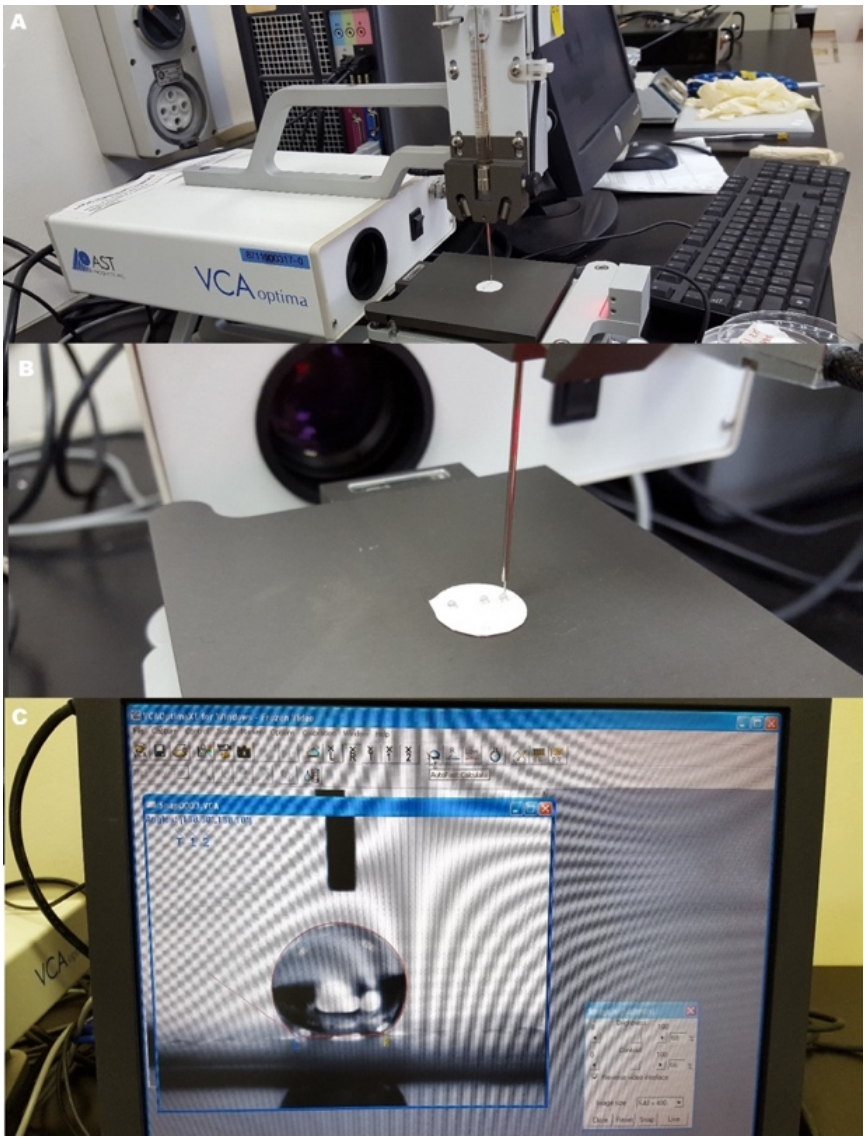

Figure 3-5 Dynamic water contact angle measurements on a VCA Optima Surface Analysis system. (B) dropping 1µl of distilled water on the surface of the mats. (C) Image obtained from VCS system allowing the measurement of water contact angle.

3.3 Plasma Treatment

Plasma treatment is typically used to modify the physical and chemical surface properties of polymers without affecting their bulk characteristics. It is thus commonly used to tailor surface adhesion and wetting properties by changing the surface chemical composition of the polymers. The application of oxygen plasma on polymers substrates has generated promising results on promoting cell growth owing to the incorporation of hydrophilic and oxygen functional group. The surface treatment for all scaffolds was conducted in a plasma chamber (Plasma Electronic PICCOLO) equipped with 13.56 MHz radio frequency

plasma generator. Plasma treatments were performed under the same condition with plasma power of 120 W, the flow rate 100 mL min^{-1} for 8 seconds under a total pressure of 20 Pa.

3.4 Morphological characterization by SEM Imaging

The scanning electron microscopy analysis of all mats was performed on a FE- SEM (FEI-QUANTA 200F, The Netherland), accelerating voltage of 15KV after sputter coating the samples with gold. The Images Analysis Software was used to estimate the average diameter for various scaffolds. To evaluate the diameter of each sample, ~100 nanofibers diameters were randomly selected and used to measure the respective diameter. For the scaffolds seeded with cells, the cells were fixed with 300 µl of 3 % of glutaraldehyde in cold PBS. Then the scaffolds were washed with distilled water to remove glutaraldehyde. Then, each well was washed for 15 min with ethanol of 30%, 50%, 75%, 90%, 100%. The wells were treated with 200 µl of hexamethyldisilazane (HMDS), left overnight in the fume hood. Each sample was coated with gold and analyzed under SEM.

3.5 Fourier- transform infrared spectroscopy (FT-IR)

The infrared spectrum was recorded using Nexus 670 intelligent FTIR spectrometer by compressing all mats scaffolds: PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI. In this study, FTIR spectroscopy was used to detect the functional group´s composition of all electrospun mats. The tested wavelength ranged from 4000 to 500 cm^{-1}.

3.6 Determination of Mechanical Properties

The determination of mechanical parameters such as Work of Failure, Failure Strain, Young's Modulus and Tensile Strength, was done following the protocol ASTMD882-02, for all the electrospun mats. To carry out the experiment was used, at ambient conditions, a tabletop tensile tester (Intron 5345, USA) using a capacity of the load cell of 10 N, see **Figure 3-6** and **Figure 3-7**. The mats were cut into rectangular strips of 1cm x 3 cm, therefore, the thickness of each sample was measured by micrometer caliper. The last step was done by placing each sample on the gripping unit of the tabletop tensile tester at a crosshead speed of 5 mm min^{-1}. The results reported are the average of 3 independent measurements.

Figure 3-6 Tabletop tensile tester.

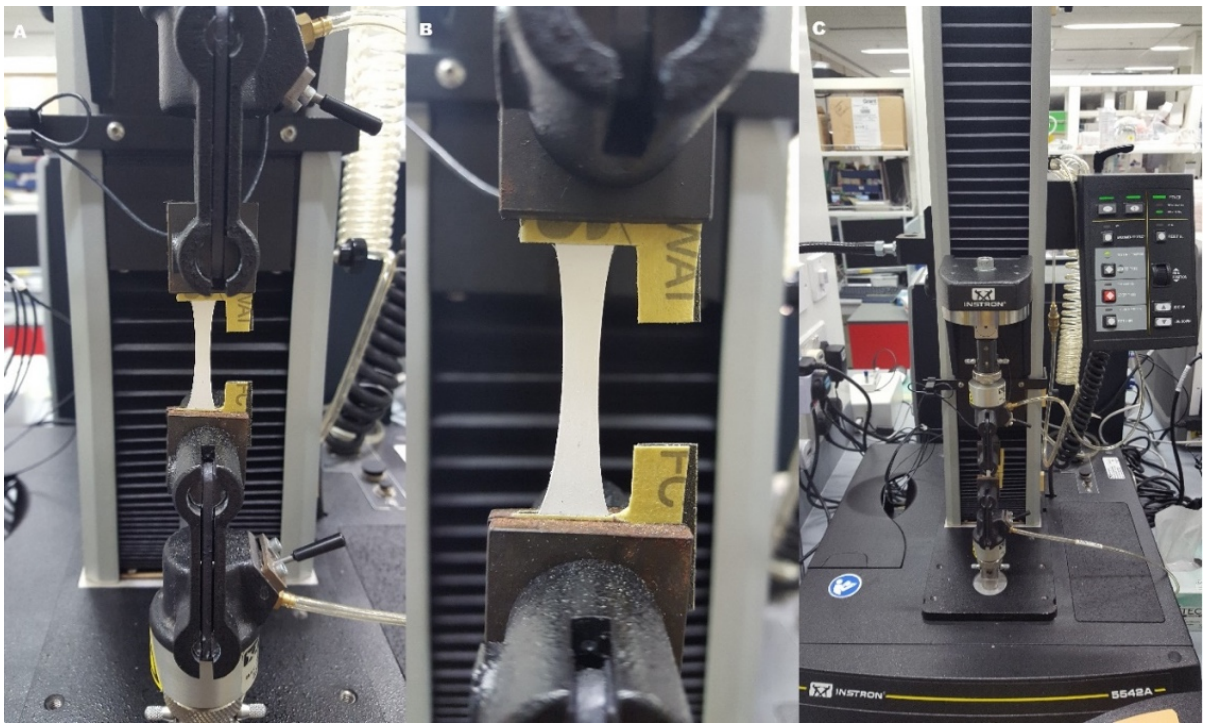

Figure 3-7 (A) Test of Mechanical properties using a capacity of the load cell of 10 N. (B) electrospun mats cut into rectangular strips of 1cm x 3 cm. (C) Failure point of electrospun mats.

3.7 Cell culture

3.7.1 Human Dermal Fibroblasts (HDF) and Murine Melanoma Cells B16

HDF and B16 cells were cultured in DMEM supplemented with 10% of FBS and antibiotics. We used 75 cm^2 cell culture flask. Both cells were incubated at 37°C in humidifier CO_2 incubator for 1 week and fed with fresh complete DMEM every 3 days. It was used trypsin- EDTA and the cells were replated after cell counting using hemocytometer. For cell seeding into the electrospun mats, all the mats were collected on 15 mm coverslips and sterilized for 1 hour under UV radiation, plated in 24 well plates, to prevent scaffolds lifting up, the coverslips were plated with a stainless steel ring. The mats were washed with 10 nM PBS (7pH) three times for 15 min each, to remove any possible residual solvent and left overnight in complete DMEM. The HDF and B16 cells were seeded at a density of 2 $x10^4$ cells $well^{-1}$ on PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI scaffolds. Tissue culture plate (TCP) served as positive controls.

3.8 CMFDA- 5-Chloromethylfluorescein Diacetate

CMFDA is a cell penetrating dye and readily cleaved by the intracellular esterases present in live cells, thus producing green- fluorescent calcein. After 3, 6 or 9 days of cell growth, the complete medium was removed from the 24-well plates and the cells were fed with DMEM medium. The scaffolds were then incubated with 20 ml CMFDA dye (final concentration 25 mM in medium) for 2h at 37°C. Thereafter, the CMFDA-medium was removed, and 1 ml complete medium was added to the cells and incubated overnight. Before imaging by confocal microscopy, cells were treated with Cytiva Cell Health Reagents for 1 h to determine cell count, nuclear morphology and cell viability. Confocal images with z-stacks were acquired with 405, 488 and 561 nm lasers excitation using Zeiss LSM800 Airyscan a Plan-Apochromat X 40/1.3 oil immersion objective lens. To quantify cell viability, images were acquired by an automated microscope INCellAnalyzer 2200 (GEHealthcare Life Sciences), 9 randomly selected fields/samples using a X 10 objective. Quantitative live/dead cell analysis of the acquired images was performed with IN Cell Investigator software (GE Healthcare Life Sciences).

3.9 Sirius Red Collagen

The fibrous scaffolds were collected on 15mm coverslips and sterilized under UV radiation for 3 hours. The scaffolds were placed in 24 well plates with stainless steel rings to avoid lifting up the scaffolds from the coverslips. Each scaffold was then washed three times with 10 mM PBS (pH 7), each wash was left for 15 minutes, aiming to remove any residual solvent. The scaffolds were finally soaked with completed media overnight. The HDF cells were seeded at the density of 2 x 10^3 cells well $^{-1}$. The plates were kept in the incubator at 37°C in humidified CO_2 for 10 days and fed with fresh complete medium every 3 days. At the days 6 and 10 after the incubation. The plates were stained for Sirius assay. The cells were fixed with 500µl of 10% of formaldehyde, then was added 200 µl of hematoxylin for one hour. Then the wells were washed 3 times of 15 minutes each with DI water, added 250µl of 0.1% Sirius red solution, which was left in the incubator for one hour. The wells were finally washed with 100% ethanol and observed under the Leica microscope for collagen release profile.

3.10 MTS Assay

Cell viability was measured using the 3-(4,5-di- methylthiazol-2-yl)-5-(3-carboxymethyl-oxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric assay. The kit (CellTiter 96s Aqueous One Solution Cell Proliferation Assay) was purchased from Promega and the µmeasurement was conducted according to the manufacturer's directions. Before the MTS testing, the culture medium was pipetted out from the 24-well tissue culture plate and 500 ml new fresh culture medium was added to every well. A hundred microliters of CellTiter 96s Aqueous One Solution Reagent was added into every well. After culturing for 4 h, the deep colored culture medium was pipetted out and added into a 96- well plate (100µl/well) and the absorbance at 490nm was recorded using an ELISA plate reader, see **Figure 3-8**. Samples with culture medium but without cells were set as a control to get background absorbance to be subtracted from the absorbance of the cell-containing wells. The overall summary of the practical work can be seen in **Figure 3-9.**

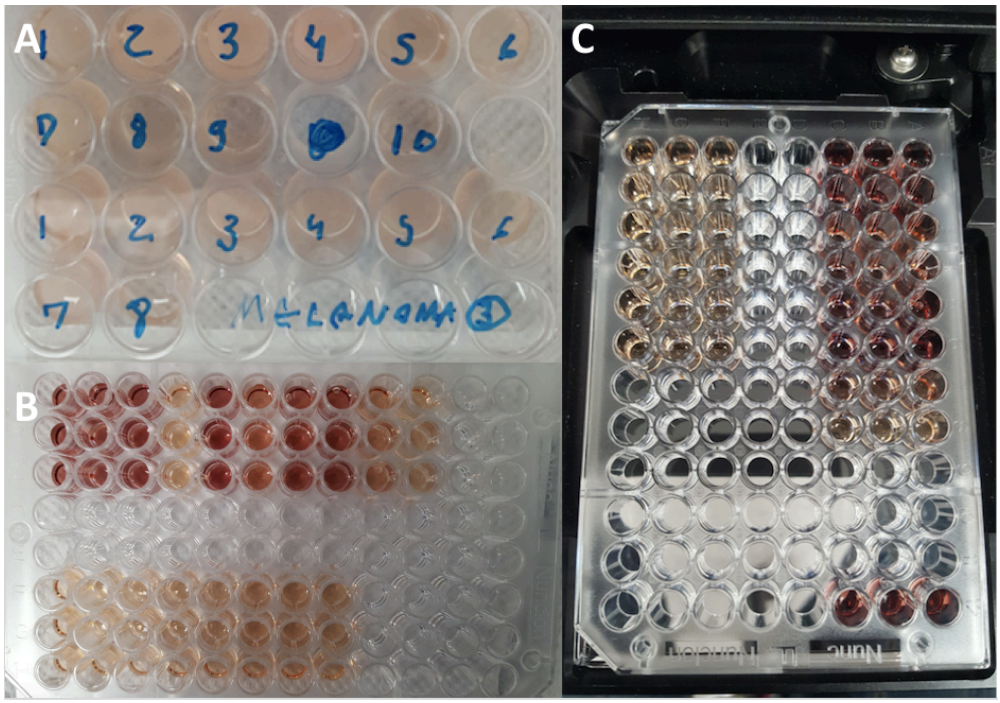

Figure 3-8 MTS Assay. A-) 24 well culture plate seeded with b16 Melanoma cells. B-) After culturing for 4 hours the colored culture with CellTiter96 was plated into 96 well plate (100µl/well). C-) Elisa plate reader at 490nm absorbance.

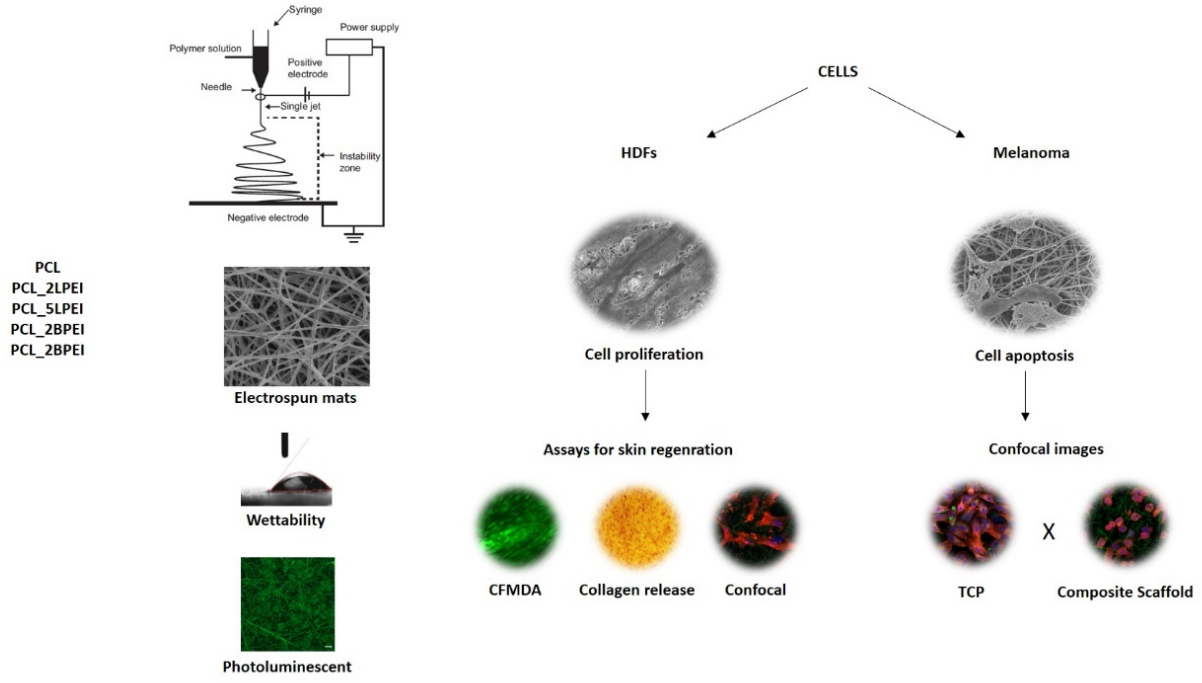

Figure 3-9 Overall summary of the present work. Electrospinning of PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI.The electrospun mats displayed excellent mechanical properties show good cells adhesion and proliferation for HDFs, and great collagen release profile, for Melanoma cells the results indicate good adhesion on the mats surface, high cytotoxicity for composites scaffolds, specially PCL_2BPEI, compared to TCP.

4 CHAPTER

RESULTS AND DISCUSSION

4.1 Morphological Analysis of Electrospun Nanofibrous Scaffolds

The SEM micrographs of the nanofibers obtained at a different concentration of LPEI and BPEI are shown in **Figure 4-1**. The images reveal that all of the fibers are continuous and random with few beads. At 1µm scale bar, it can be seen that the overall parts of the obtained fibers all have good fibrous morphology, suggesting that blending of PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI does not destroy the fibrous structure, it is not presented any collapse nor breakage. The diameters and morphologies of electrospun nanofibers were strongly affected by LPEI and BPEI concentration and type of polymer used to blend with PCL. For LPEI mats, it can be seen that the average of electrospun nanofiber decreases with an increase of LPEI in the solution. The standard deviation of all diameters found in each electrospun mats was calculated. Accordingly, the fiber diameter decreased from 331±74 nm for neat PCL fibers (which were named as PCL in the experiment) to 258 ±109 nm for PCL_5LPEI. This is because of the cationic characteristics of LPEI enhanced the conductivity solution. When analyzing PCL blended with BPEI, the fiber diameter distributions demonstrate that the diameter of the average fibers diameter increases with increased concentration of BPEI. The diameter was increased from 331±74 for neat PCL to 419 ± 233 nm for PCL_5BPEI. From SEM observations, the electrospun fibers showed numerous porous structures among the fibers, which could improve the contact cell to scaffold mats, helping the adhesion process.

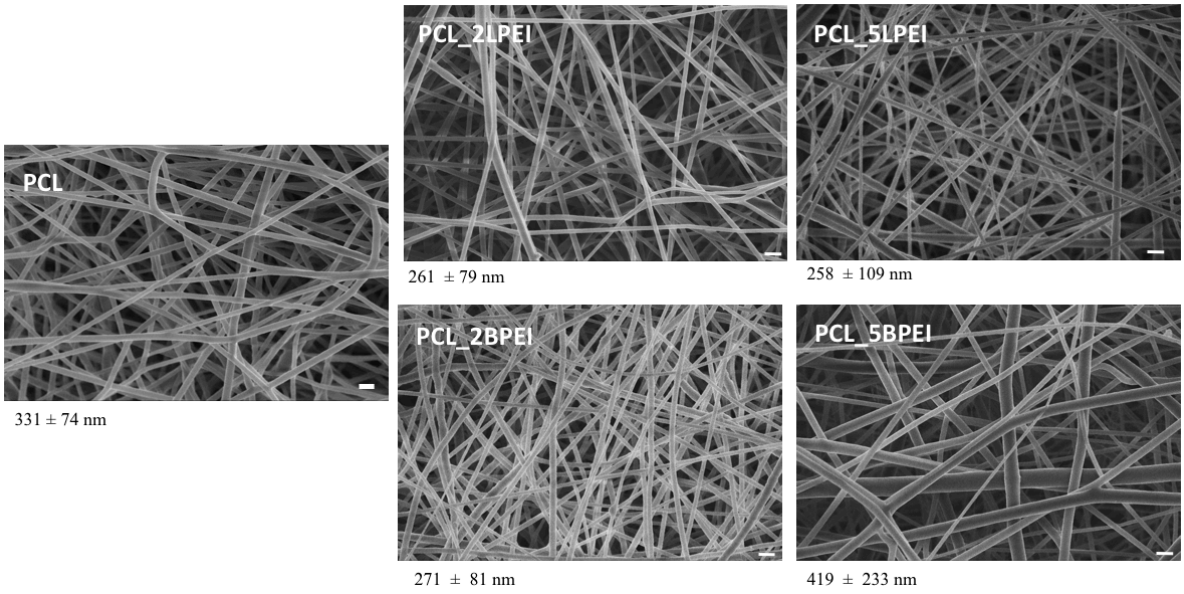

Figure 4-1 Scanning electron microspe (SEM) images of all nanofibers. Scale bar = 1µm

4.1.1 Wettability

The surface properties of polymer scaffolds play a major role in the interaction between cells and the matrix. Among many factors influencing the surface chemistry of scaffolds, the balance of hydrophilicity and hydrophobicity is very important for cell adhesion. Water contact angle assay can be used to reflect the hydrophilicity of the scaffolds, since cells may attach and proliferate less well on scaffolds having a wettability that is higher than 90°. All the scaffolds prepared in this study showed hydrophobic behavior with a contact angle higher than 87°, as we can observe in **Figure 4-2** for non-treated mats. To ameliorate the hydrophilic properties, the scaffolds undergone a plasma treatment for 8s. The observed results after plasma treatment are shown in **Figure 4-2**. After plasma treatment, we could observe the improvement of hydrophilic properties. The contact angles were decreased from 130° to 32°, 136° to 31°, 135° to 36, 136° to 31°, 87° to 31, for neat PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI PCL_5BPEI, respectively. The hydrophilic properties of the nanofibrous scaffolds are directly proportional to the rate of water absorbance. The improved wettability is expected to be beneficial for cell attachment.

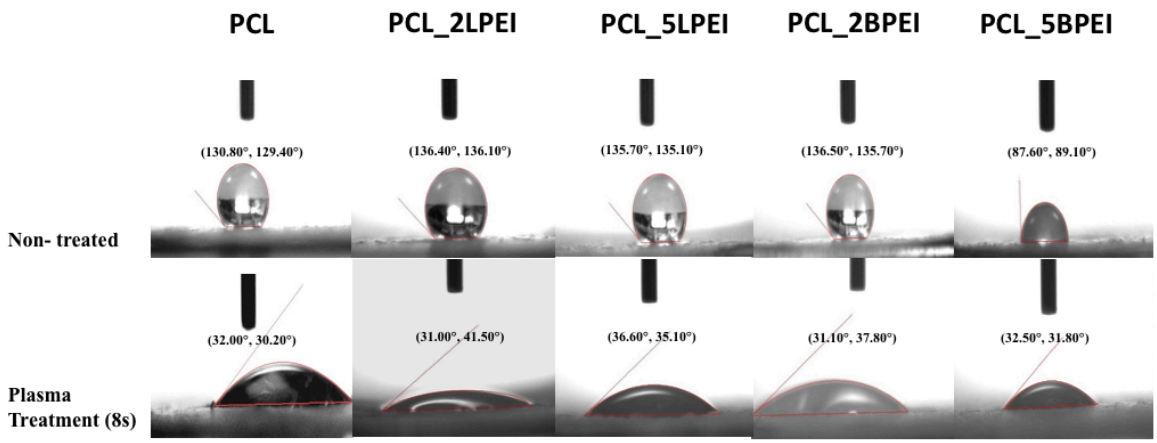

Figure 4-2 Water contact angle assay of all mats, namely PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI.

4.1.2 Characterization by FT-IR

FTIR analysis was used to detect the possible changes in the chemical structure of the electrospun materials, **Figure 4-3**. Regarding PCL, spectra showed that the main absorption of the PCL such as asymmetric and symmetric stretching of the methylene groups (2944 and 2865 cm^{-1}), banding at 1294 cm^{-1}, corresponding to C-O stretching of the crystalline phase of the PCL and 1242 and 1189 cm^{-1} associated with asymmetric (C-O-C) and symmetric (OC-O) stretching, respectively, and stretching of the ester carbonyl (O-C=O) at 1726 cm^{-1}. A close inspection of PCL mats spectra showed that the presence of a small band close to 3439 cm^{-1}, attributed to O-H stretching vibration and the 1726 cm^{-1} peak exhibits a small shoulder located at 1708 cm^{-1}, which can be associated with COOH groups. The spectra show characteristic absorption peaks of PEI at 1580 and 1460 cm^{-1}, in the linear PEI, the amide group I peak is at 1648 cm^{-1}, and 1552 cm^{-1} corresponds with the amide II peaks. The branched PEI shows it characteristic absorption bands at about 3440 and 1654 cm^{-1} assigned to the stretching vibration of the group $-NH_2$.

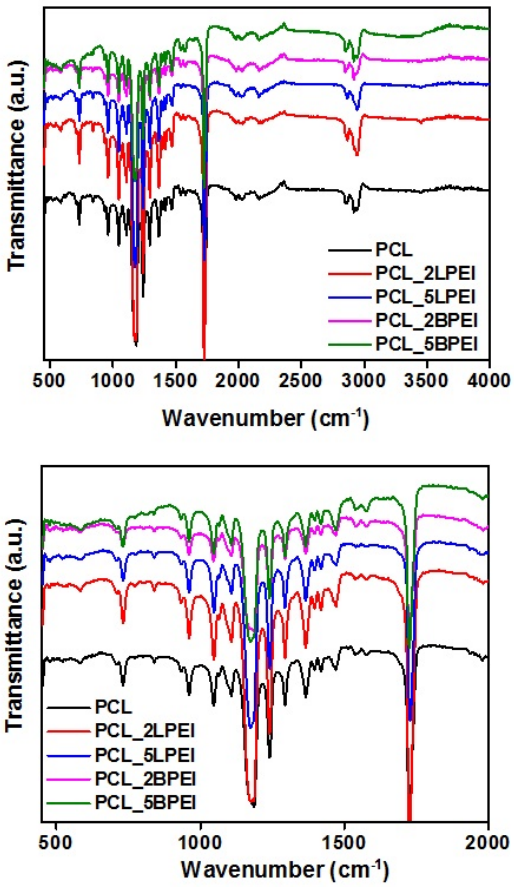

Figure 4-3 FT-IR characterization of electrospun mats prepared under various condition.

4.1.3 Mechanical Properties

The mechanical properties of PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI nanofibrous scaffolds are shown in **Table 4-1**, and the scaffolds revealed a characteristic semilinear stress-strain curve as shown in **Figure 4-4**. The Young's modulus obtained for PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, and PCL_5BPEI were 7.2±2.0 MPa, 15.57±6.8 MPa, 15.18±3.97 MPa, 5.97±3.27 MPa, 7.53±3.96 MPa, respectively. The tensile properties of PCL_5LPEI and PCL_2LPEI, were higher when compared to the PCL, and lower for PCL_5BPEI and PCL_2BPEI. The observed results proved that the increase of concentration of PEI polymer from 2 to 5% results increased tensile stress. The results show that PCL blended with LPEIs have better mechanical properties to the scaffolds than BPEIs scaffolds, which have almost the same mechanical properties as PCL. The blend of PEIs may reduce the tensile break of nanofibrous scaffolds observed in **Figure 4-4**. Mechanical stability of the scaffolds is desirable to provide cell growth and proliferation and degrade itself while the patient natural ECM starts regenerating the injured body sites.

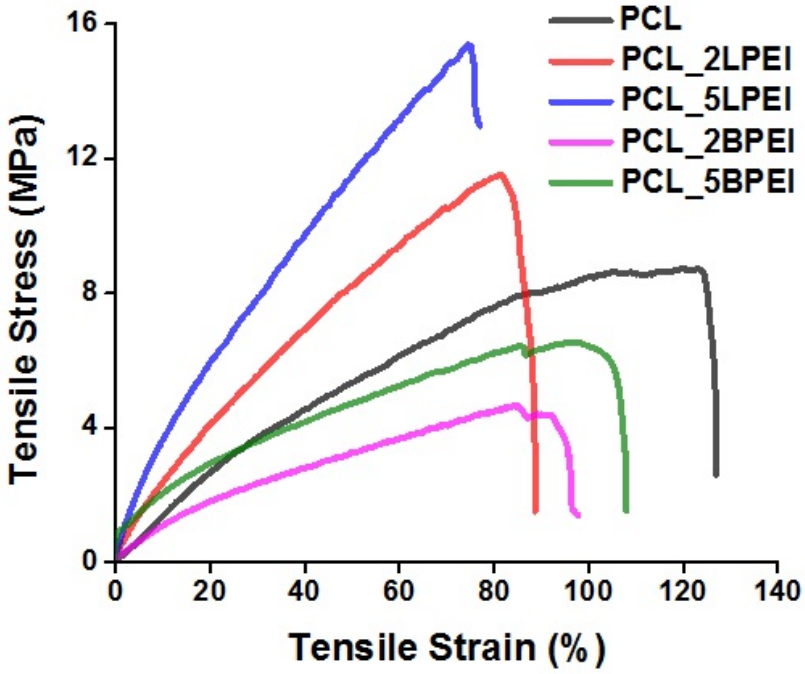

Figure 4-4 Tensile stress-strain curves of PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, and PCL_5BPEI. Abbreviations: PCL: -poly-(ε-caprolactone), PCL_2LPEI: poly-(ε-caprolactone) blended with 2% of linear polyethyleneimine, PCL_5LPEI: poly-(ε-caprolactone) blended with 5% of linear polyethyleneimine, PCL_2BPEI: poly- (ε-caprolactone) blended with 2% of branched polyethyleneimine, PCL_5BPEI: poly-(ε-caprolactone) blended with 5% of branched polyethyleneimine.

Table 4-1 Mechanical Properties of mats prepared under various conditions. The table shows Tensile strength (MPa), Failure Strain (%), Young´s Modulus (MPa) and Work of Failure (MJm^{-3}).

Sample	Tensile Strength (MPa)	Failure Strain (%)	Young´s Modulus (MPa)	Work of Failure (MJm^{-3})
PCL	7.8 ± 1.5	135.8 ± 8.2	7.2 ± 2.0	6.4 ± 1.0
PCL_2LPEI	11.1 ± 4.5	78.2 ± 12.4	15.6 ± 6.8	5.1 ± 2.0
PCL_5LPEI	12.5 ± 3.0	82.4 ± 4.5	15.2 ± 4.0	2.4 ± 0.6
PCL_2BPEI	4.0 ± 1.0	98.3 ± 3.8	5.9 ± 3.3	2.4 ± 0.6
PCL_5BPEI	4.2 ± 2.1	87.3 ± 33.2	7.5 ± 4.0	2.6 ± 2.1

4.2 Evaluation of Biocompatibility of the Scaffolds for Human Dermal Fibroblast

4.2.1 Scanning Electron Microscopy – SEM

The design of scaffolds involves certain specific requirements of the scaffolds for tissue/ skin reconstruction, it should have adequate pore size for seeding the cells, biodegradability, and diffusability through the matrix. We proposed PCL, -poly-(ε-caprolactone); PCL_2LPEI, poly-(ε-caprolactone) blended with 2% of linear Polyethyleneimine; PCL_5LPEI, poly-(ε-caprolactone) blended with 5% of linear Polyethyleneimine; PCL_2BPEI, poly- (ε-caprolactone) blended with 2% of branched Polyethyleneimine; PCL_5BPEI, poly-(ε-caprolactone) blended with 5% of branched Polyethyleneimine fibrous mats for skin tissue engineering applications and hence fibrous mats were evaluated for its suitability with human dermal fibroblasts. Cell attachment study was carried using human dermal fibroblast. HDFs cells show good attachment, spreading and proliferation. We used tissue culture plate as control, followed by PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI. In all mats is possible to observe the attachment of cells. It is important to note that we conducted the study for one week, changing medium every three days. In **Figure 4-5** and **Figure 4-6**, we can observe the cell proliferation for day 4 and day 7, respectively. The images show the HDFs dispersion and morphology within the fibrous mats observed by SEM. By day 7, cells covered the surface of the 3D porous structure, and closure to the interconnected pores. The HDFs on PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI expressed good cell-cell contact with almost no exposed fibers, the improved biocompatibility and hydrophilicity of the electrospun mats after plasma treatment, as previously mentioned, act to promote proliferation and migration of the cells. The cell adhesion on a surface is strongly influenced by the balance of hydrophobicity and hydrophilicity. The cells can adhere, grow and spread more easily on the moderate hydrophilic substrate than on very hydrophilic or hydrophobic ones. This is required for skin tissue engineering and wound healing as HDFs need good cell-cell contact and strong cell ECM interaction.

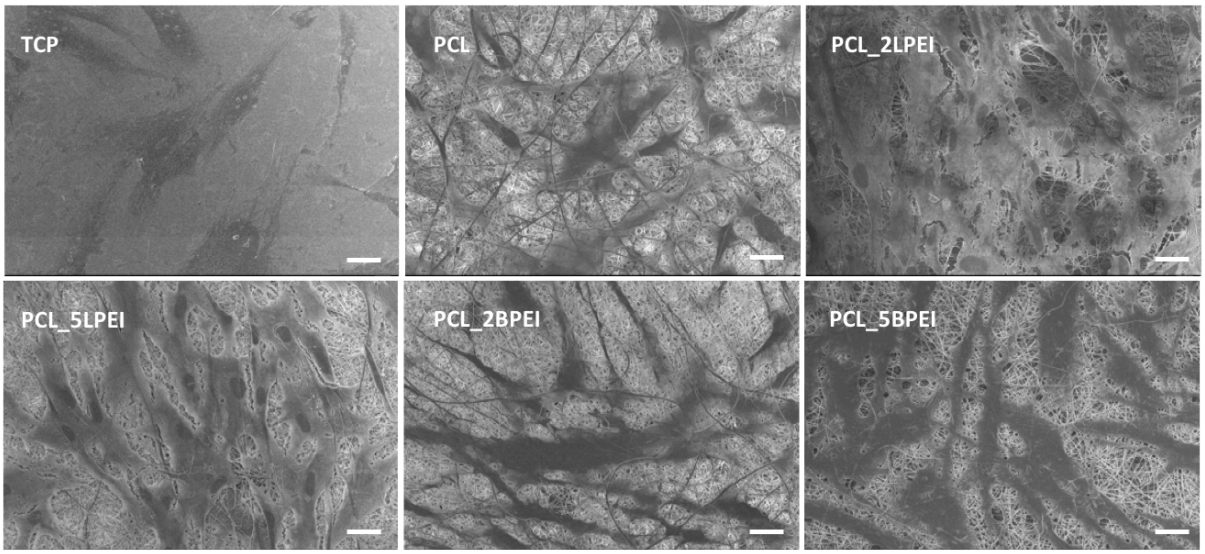

Figure 4-5 Scanning electron microscope (SEM) images show the biomaterial-cell interaction on the day 1. PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI and TCP on day 4. Scale bar = 1µm. Abbreviations: PCL: -poly-(ε-caprolactone), PCL_2LPEI: poly-(ε-caprolactone) blended with 2% of linear Polyethyleneimine, PCL_5LPEI: poly-(ε-caprolactone) blended with 5% of linear Polyethyleneimine, PCL_2BPEI: poly- (ε-caprolactone) blended with 2% of branched Polyethyleneimine, PCL_5BPEI: poly-(ε-caprolactone) blended with 5% of branched Polyethyleneimine.

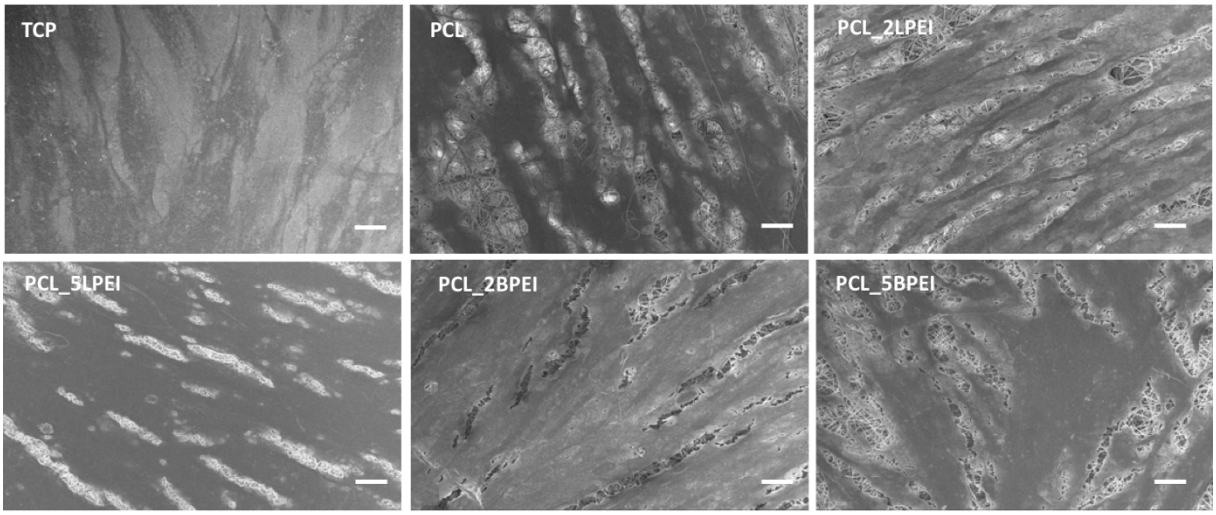

Figure 4-6 Scanning electron microscope (SEM) images show the biomaterial-cell interaction on the day 6. PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI and TCP on day 7. Scale bar = 1µm. Abbreviations: PCL: -poly-(ε-caprolactone), PCL_2LPEI: poly-(ε-caprolactone) blended with 2% of linear Polyethyleneimine, PCL_5LPEI: poly-(ε-caprolactone) blended with 5% of linear Polyethyleneimine, PCL_2BPEI: poly- (ε-caprolactone) blended with 2% of branched Polyethyleneimine, PCL_5BPEI: poly-(ε-caprolactone) blended with 5% of branched Polyethyleneimine.

4.2.2 HDFs Cell Viability- MTS Assay

Cell viability was monitored after 1,4, 7 and 10 days of incubation time by the colorimetric MTS assay 3-(4,5- dimethylthizol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4- sulfophenyl)-2H-tetrazolium, inner salt (CellTitel 96 Aqueous one solution cell proliferation assay). The HDFs viability was first revealed on day 1, after 24 hours of incubation time. In this experiment, we used tissue culture plate (TCP), as the negative control and cells treated with Nocodazole as the positive control. All mats PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI, when compared to TCP show no cytotoxicity after 24 hours, the well treated with Nocodazole presents high toxicity, this was thought to serve as a comparison for this viability assay. Moreover, their viability on PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI did not vary during the observation, the same behavior has been observed for HDFs seeded on TCP, see **Figure 4-7**.

After we observed no cytotoxic for the mats, we follow-up the HDFs cells during 10 days after seeding. The follow up viability is presented in **Figure 4-8**.

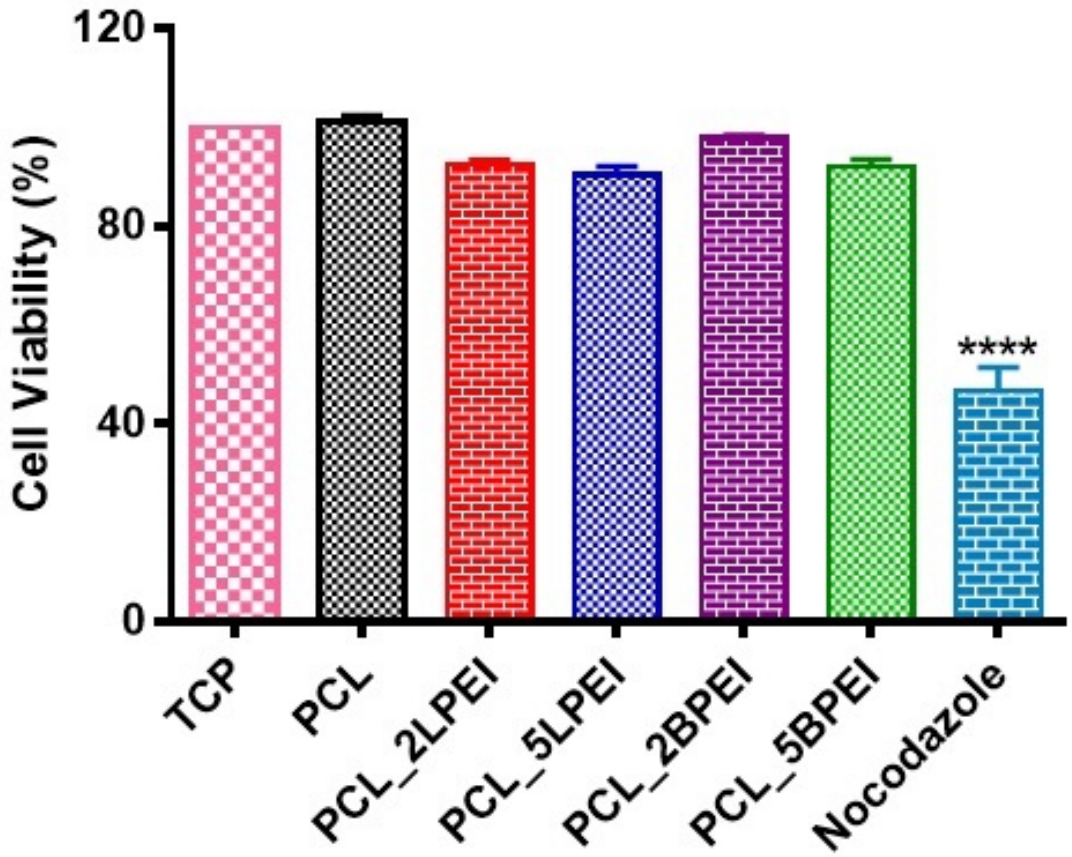

Figure 4-7 MTS assay reading of electrospun mats after 24 hours of incubation time of human dermal fibroblast (HDF) cells. (****, represents high cytotoxicity). Absorbance Index 490 nm.

On day 4 it is possible to observe higher cell viability on TCP, followed by PCL, PCL_2LPEI, PCL_2BPEI, the cell viability for PCL_5LPEI and PCL_5BPEI were almost the same value. On day 7, although we variation of cell growth on all seeded mats, none of them are toxic because cell viability is still present. It is possible to observe, better cell viability on PCL, followed by TCP and PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, and PCL_5BPEI. Surprisingly on day 10, the viability on PCL mat decreased compared to day 7, the PCL_2LPEI reaches almost the same cell viability as TCP, followed by PCL_2BPEI and PCL5LPEI this might be too high cell proliferation and attachment to the surface. In the same graphic, we can observe that PCL _5BPEI had lower viability rate compared to the others mats, we cannot assure that this lower proliferate indicates cytotoxicity, on the contrary, toxic biomaterial do not present cell growth viability as PCL_5BPEI presented. From this data, we can state that none of the mats are toxic for HDFs cells.

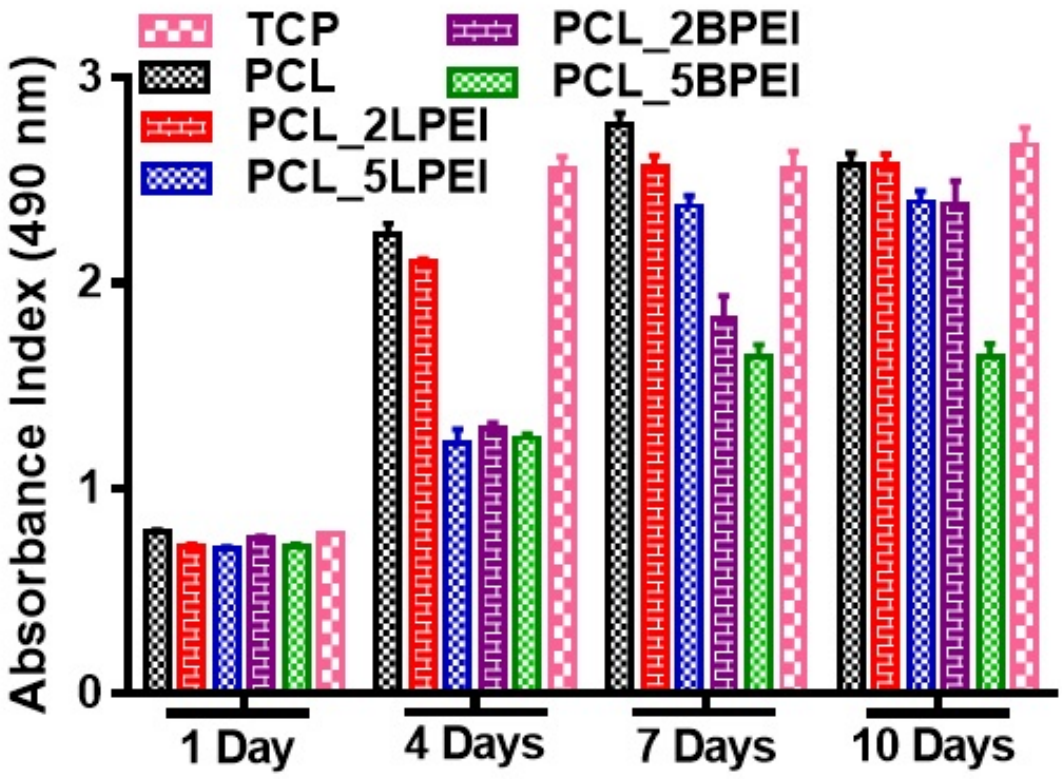

Figure 4-8 MTS assay reading of electrospun mats after 1, 4, 7 and 4 days of incubation time of human dermal fibroblast (HDF) cells. Absorbance Index 490 nm.

4.2.3 CFMDA

We examined biological properties of Human Dermal Fibroblast, seeded into PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI and TCP scaffolds, in terms of assessing their cytocompatibility to enhance proliferation and cell adhesion. The cells were seeded on PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI mats. Cells seeded on the tissue culture plate (TCP) served as control. After four days post seeding (p.s.) of HDF on various scaffolds, the cells were stained with green *CMFDA* (5-Chloromethylfluorescein diacetate) to obtain semiquantitative information about living and dead cells. All the scaffolds studied displayed no discernible cytotoxicity to HDF cells, therefore, confirming the biocompatibility of all scaffolds for HDF. The confocal images are shown in **Figure 4-9**. Interestingly, the scaffold 5_LPEI has shown a decreased HDF proliferation, although, no cytotoxicity is presented.

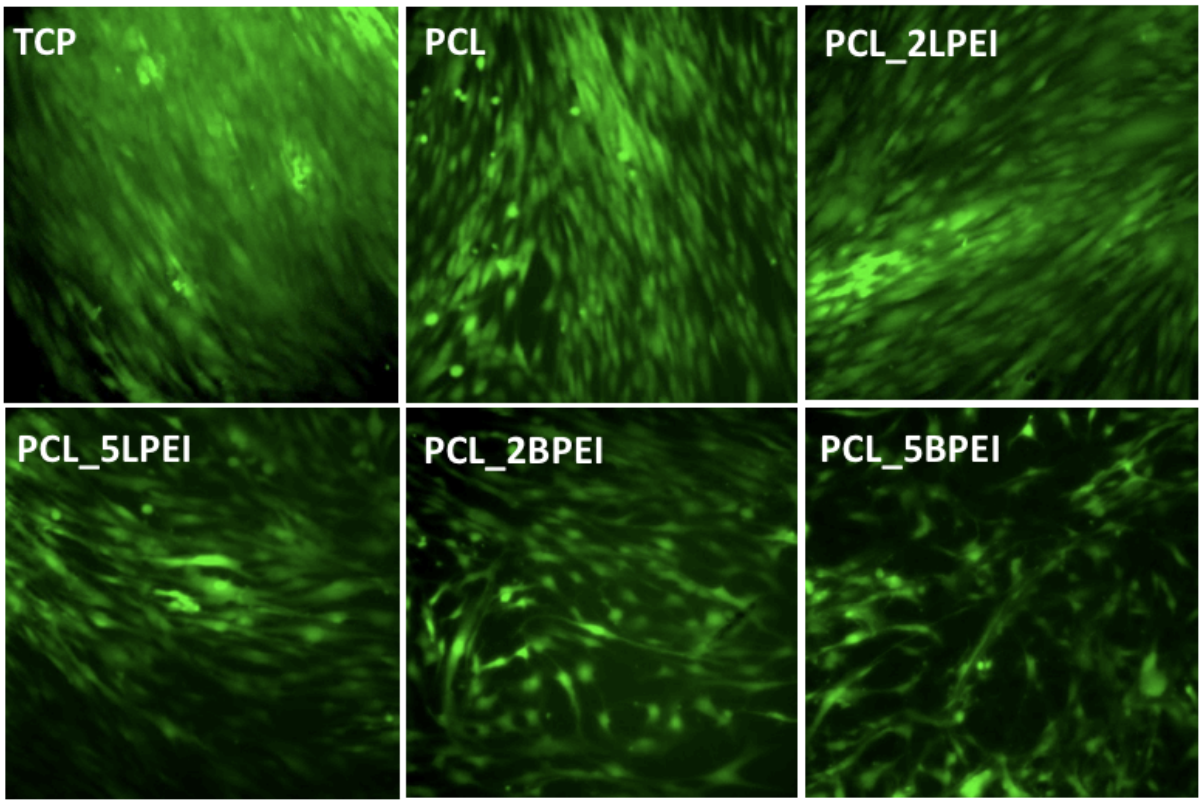

Figure 4-9 CMFDA confocal imagens on TCP, PCL 10%, PCL 10%_LPEI2%, PCL 10%_LPEI 5%, PCL 10%_ BPEI 2%, PCL 10%-BPEI 5%. Scale bar = 20µm. Abbreviations: PCL: -poly-(ε-caprolactone), PCL_2LPEI: poly-(ε-caprolactone) blended with 2% of linear Polyethylenimine, PCL_5LPEI: poly-(ε-caprolactone) blended with 5% of linear polyethylenimine, PCL_2BPEI: poly- (ε-caprolactone) blended with 2% of branched Polyethylenimine, PCL_5BPEI: poly-(ε-caprolactone) blended with 5% of branched Polyethylenimine, TCP: tissue culture plate.

4.2.4 Sirius Red Collagen Assay

In the ECM formation, fibroblasts are essential in their tissue of residence. In the dermis, they can release collagen and other important components of the ECM. During the wound healing process, the fibroblasts are essential for wound closure synthesis of the provisional as well as remodeled scar matrix. Here, we tested whether human dermal fibroblast cultured in PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI electrospun scaffolds could release collagen on these subtracts. In **Figure 4-10**, is possible to observe the collagen expressed in human dermal fibroblast cells, after 6 days of incubation time. It results in a layer of decellularized extracellular matrix produced by the cells. This extracellular matrix was stained for collagen with Sirius red. As we can observe, the reddish color in the followed figure represents the collagen expression in HDFs. Collagen deposition was higher into PCL_2LPEI, PCL_5LPEI, PCL_2BPEI subtracts than TCP, PCL, and PCL_5BPEI. The optical microscopy images of scaffolds stained with Sirius Red Collagen for 6 days support the above data, where high intensity of staining materials was observed on PCL_2LPEI, PCL_5LPEI, PCL_2BPEI electrospun scaffolds displaying an overall enhancing effect in collagen expression in HDFS cells and proving these scaffolds as an indispensable method for the fabrication of superior nanofibrous scaffolds for skin tissue engineering.

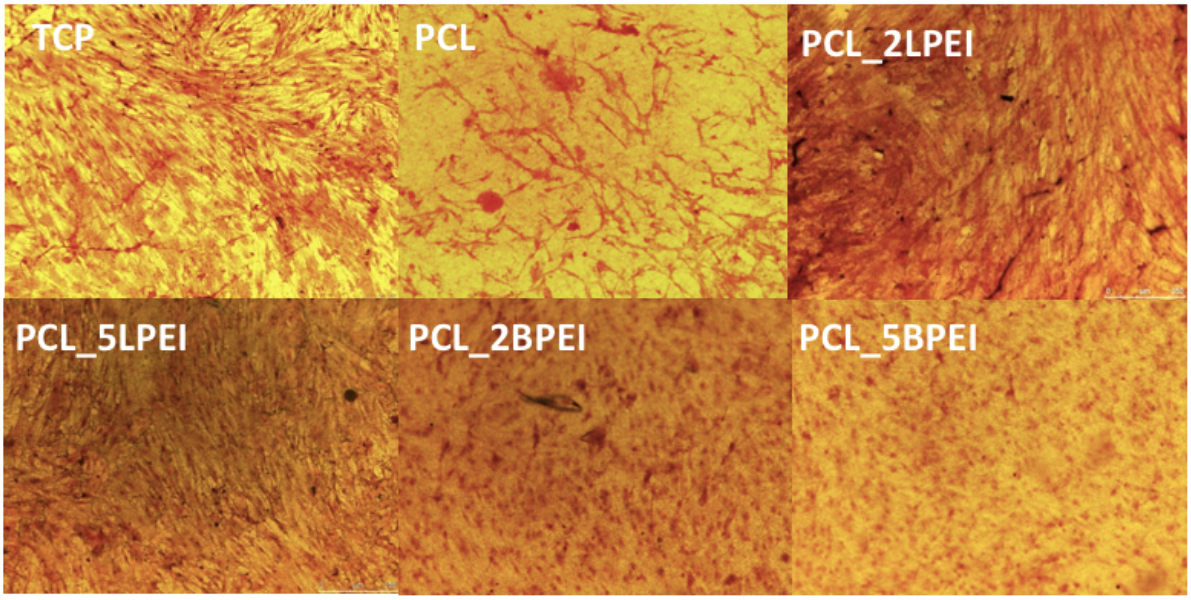

Figure 4-10 Sirius red collagen assay, observation of collagen release on the day 6. Scale bar = 50μm.

4.3 Evaluation of Cytotoxicity for Melanoma Cells

4.3.1 Scanning electron microscopy – SEM

The design of scaffolds involves certain specific requirements of the scaffolds for melanoma (B16) cytotoxic effect, it should have adequate pore size for seeding the cells, biodegradability, and diffusability through the matrix. We proposed PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI fibrous mats as cytotoxic functional biomaterials for melanoma as an alternative approach for melanoma treatment, hence fibrous mats were evaluated for its cytotoxicity activity with B16 melanoma cells. Cell attachment study was carried using B16 cells, the SEM micrographics are presented in **Figure 4-10.** B16 cells show good attachment, spreading and proliferation. We used tissue culture plate as control, followed by PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI. In all mats is possible to observe the attachment of the cells. The images presented in **Figure 4-10,** were taken 24 hours after seeding. The images show B16 cells dispersion and morphology within the fibrous mats observed by SEM. TCP presents the cell morphology without any treatment, only medium. Differently than the image from TCP, in all mats the B16 cells grow in a different manner. The morphological image of B16 on PCL_5LPEI have the same morphology that TCP. On the mats PCL, PCL_2LPEI and PCL_2BPEI the B16 cells do not present the wounded morphology of health B16 melanoma cells, it is possible to observe that the cells are flatted and spread out along the fibers. In PCL_5BPEI SEM image, is clearly noted the degradation of cell the membrane, although still attached to the surface of the mats. As mentioned previously the cell adhesion on a surface is strongly influenced by the balance of hydrophobicity and hydrophilicity. The cells can adhere, grow and spread more easily on the moderate hydrophilic substrate than on very hydrophilic or hydrophobic ones. Cell attachment is required for drugs delivery system.

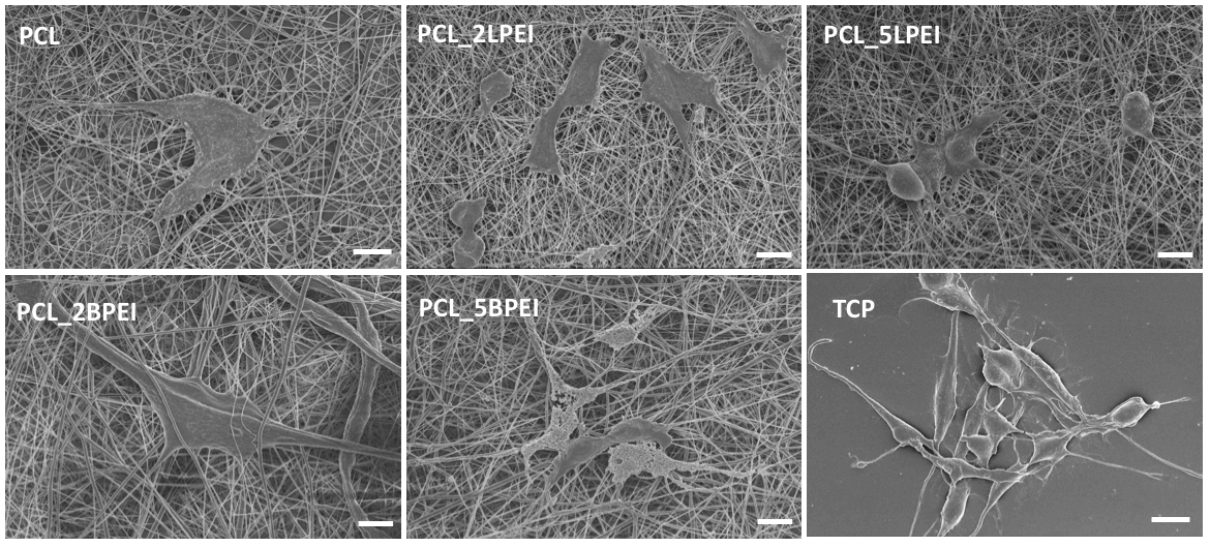

Figure 4-11 Morphology of B16 melanoma cells seeded on various mats. Representative SEM image showing features of B16 after 24 hours p.s. onto PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI. Scale bar = 10 µm.

4.3.2 Melanoma Cell Viability- MTS Assay

Cell viability was monitored for 24 hours of incubation time by the colorimetric MTS assay 3-(4,5-dimethylthizol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4- sulfophenyl)-2H-tetrazolium, inner salt (CellTitel 96 Aqueous one solution cell proliferation assay). In this experiment we used tissue culture plate (TCP), as the negative control and cells treated with Doxorubicin, which is a potent anticancer drug, serving as positive control. When compared to TCP, the mats PCL, PCL_2LPEI, PCL_5LPEI, presented good cell viability, which is the contrary of what we proposed to do as an alternative for anticancer melanoma approach. On the other hand, the mats PCL_2BPEI and PCL_5BPEI had the higher anticancer activity when compared to our positive control Doxorubicin. This is an indication that from all mats developed, the ones that have better cytotoxic effect for the B16 cells, are the PCL loaded with 2 and 5% of BPEI, it can be observed in **Figure 4-12**.

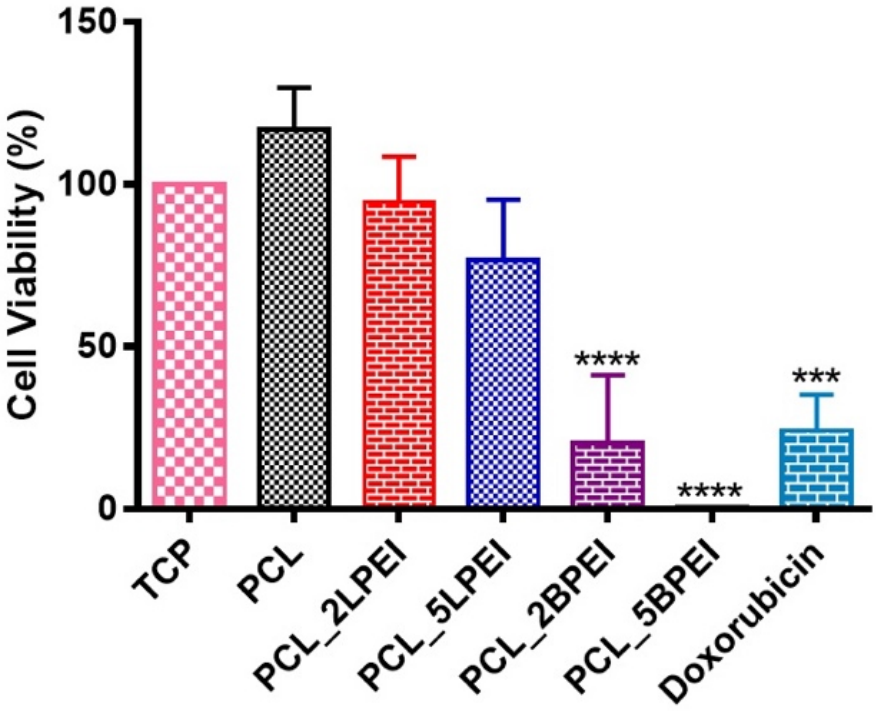

Figure 4-12 MTS assay reading of electrospun mats after 24 hours of incubation time of murine melanoma cells B16. (****, and *** represents high cytotoxicity). Absorbance Index 490 nm.

4.4 Confocal microscopy

To gain insights of cell morphology, confocal images of the melanoma cells cultured on various scaffolds are shown **Figure 4-13**, 24 hours after seeding the cells. The cells were labeled with Hoechst and Phalloidin Rhodamine Actin, for better visualization of the nucleus and membrane of the cells respectively. By taking advantage of the photoluminescent properties of the scaffolds, we used confocal microscopy to confirm the integrity of the fiber mats after 24 hours in the cell culture. It is possible to observe that the multiple layers for TCP, the cells had a normal proliferation and growth, however, we had a different outcome for PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, and PCL_5BPEI, the images suggest cytotoxicity for the cells, the membrane becomes rounded, which indicates cell death. Interesting, in our study, we did not use any green stain, which was observed on the layer of the scaffolds for PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, and PCL_5BPEI, this suggests that the melanin produced by melanoma cells give green fluorescent color, this is not observed in TCP, which served as control. In addition to that, the fluorescent signals from the cells confirmed the stability of the mats in the culture media.

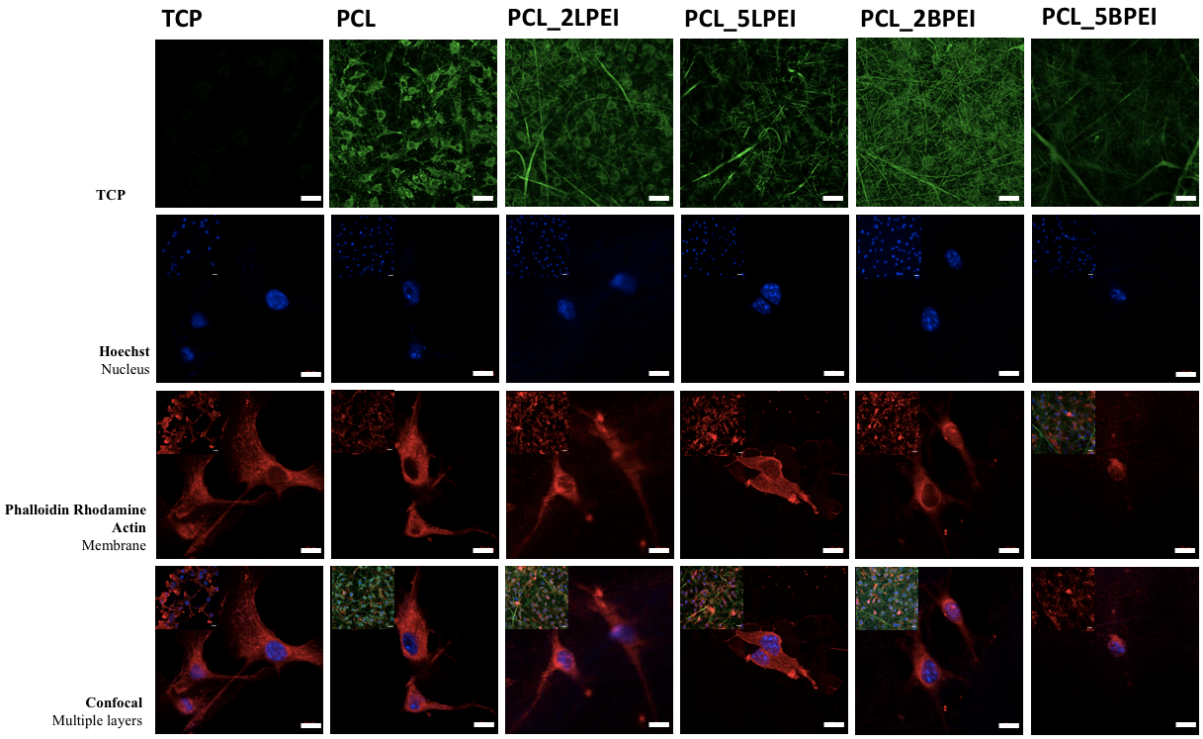

Figure 4-13 Confocal images of electrospun mats seeded with murine melanoma cells B16 after 24 hours of incubation time. Mats were stained with Hoechst, Phalloidin Rhodamine Actine, then overlayered.

Next, we performed the same study for HDFs 24 hours after seeding the cells. The cells were analyzed with Hoechst and Phalloidin Rhodamine Actin, to have a better insight of the nucleus and membrane of the cells respectively. It is possible to observe that nontoxicity is presented in the mats, due to the normal proliferation rate and morphology of cells membrane and nucleus of HDFs seeded in PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI **Figure 4-14**. As a comparison on the lower side, we have the melanoma confocal images, giving us a clear picture of the toxicity presented in the scaffolds as mentioned above for the control the proliferation of melanoma cells.

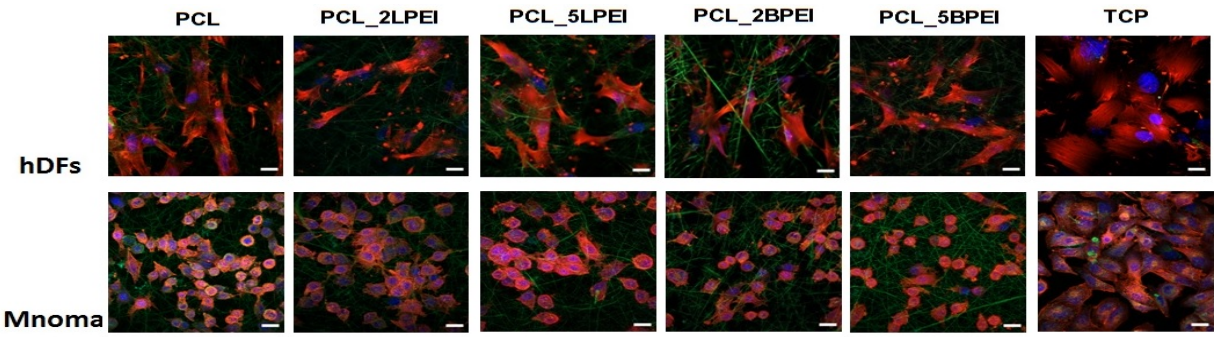

Figure 4-14 Confocal images of electrospun mats seeded with human dermal fibroblasts (hDFs) and murine melanoma cells B16 (Mnoma) after 24 hours of incubation time. Abbreviations: PCL: -poly-(ε-caprolactone), PCL_2LPEI: poly-(ε-caprolactone) blended with 2% of linear Polyethyleneimine, PCL_5LPEI: poly-(ε-caprolactone) blended with 5% of linear polyethyleneimine,

PCL_2BPEI: poly- (ε-caprolactone) blended with 2% of branched Polyethyleneimine, PCL_5BPEI: poly-(ε-caprolactone) blended with 5% of branched Polyethyleneimine, TCP: tissue culture plate.

Protein kinase C (PKC) family of serine/threonine was one of the first protein kinases to be identified. It is a heterogeneous group of enzymes integrating and receiving signals in both normal melanocytes and melanoma pathology. The chromatographic purification of PKC shows that this activity was composed of at least three distinct species, designated as α, β and γ isotypes. Nowadays, it is known that PKC isotypes of mammalians are superfamily comprising twelve distinct genes. The members of mammalian, PKC, superfamily play an important key on regulatory roles in cellular processes, it has a wide range, from fundamentals autonomous activities such proliferation to cellular memory. Comparative analysis over the last few years have provided insights and defining a number of regulatory elements in PKC which confer to each isotype specific activation signals and location.

The alteration of PKC enzyme activation and expression contribute directly to the malignant phenotype of melanoma in both the tumour suppressive and oncogenic roles. These enzymes are involved in a varied array of the biological process including, migration, cell polarity, proliferation, differentiation, and apoptosis. PKC enzymes have profound influences on actin cytoskeleton organization and PKCα, which have been linked to melanoma invasion, see **Figure 4-15**. Thus, activation and/or alteration in the expression of PKCα cooperate with β3 subunit signalling, this can disrupt stable cell-matrix adhesion and facilitate melanoma invasion. The changes in integrin expression and signalling are important to melanoma invasion and metastasis. The high expression of the subunit β3 is associated with melanoma invasion and progression.

PKC activation inhibits the growth of melanoma cells while stimulating proliferation of normal melanocytes cells. The inhibitory growth effect of PKC activation is due to some PKC enzymes possess tumour suppressive functions in melanoma cells (Mason et al., 2010).

An agonist is a chemical that binds to a receptor and activates the receptor to produce a biological response. PKC agonist with differential desensitization/activation specificities holds a promise to the development of PKC based therapeutics for melanoma, by having multiple mechanisms of actions, such as inducing expression of their target PKC enzyme or disrupting tumour vasculature.

Multidrug resistance (MDR) is an important hindrance for cancer therapy. Polymeric gene carriers have attracted attention for potential application in the fled of gene therapy, especially for cancer therapy. One of the most important approach to avoid side effects is cancer-specific gene delivery. As we know, protein kinase (PKs) plays an important role in regulating various cellular functions, so, dysregulation of specific PKs activity is closely associated with many diseases, including cancer. As mentioned previously, there are various PKs, among them, the PKCα is reported as a suitable marker to distinguish carcinogenic cell from healthy cells, once that in tumour cells the PKCα activity is much higher than in healthy cells. The buffering capacity of the polyethyleneimine main chain results in polyplexes efficient escape from the endosome, showing a clear-cut response towards PKs activity. LPEI and BPEI based carries can be generally applicable to any kind of protein kinase, which is specifically activated in disease cells.

As LPEI and BPEI can target PKCα which has more intense activity in malignance cells and less activity in healthy cells, we can observe high cytotoxicity for B16 melanoma cells, and non-cytotoxicity to human dermal fibroblast cells, as shown in confocal **Figure 4-14**.

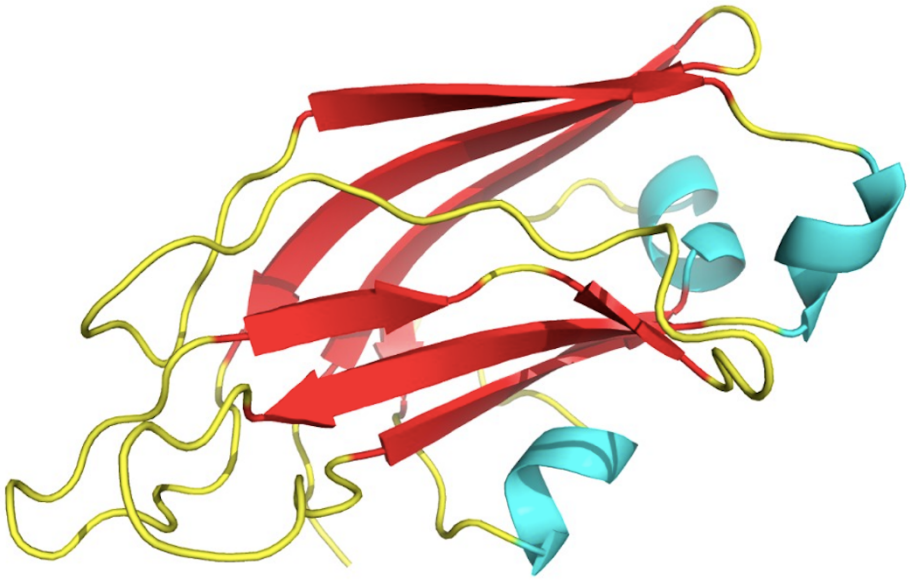

Figure 4-15 Graphical representation of Protein kinase C, α subunit.

5 CHAPTER

FINAL CONSIDERATIONS

5.1 Conclusions

In this doctoral thesis, we proposed the conception and the development of new electrospun mats, aiming high cytotoxicity to melanoma carcinogenic cells B16, with regenerative properties for ECM, achieving wound healing and skin regeneration by collagen release.

The scaffolds developed were produced by biodegradable polymers, approved by the Food and Drug Administration (FDA- U.S.). After working in this process, we come to the conclusion that:

The SEM of electrospun mats obtained from neat PCL and PCL blended with different concentration of LPEI and BPEI, both blended at concentrations of 2 and 5%, reveal that all fibers are continuous and random with few beads, having good fibrous morphology, suggesting that blending PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI does not destroy the fibrous structure, not presenting any breakage nor collapse. The diameters and morphologies of all electrospun mats were strongly affected by the concentration of LPEI and BPEI used to blend into PCL solution. The diameter was increased from 331±74 for neat PCL to 419 ± 233 nm for PCL_5BPEI. Also, the SEM observations, lead us to conclude that all electrospun mats developed showed innumerous porous structure among the fibers, this characteristic could enhance the surface mats to cell interaction, helping cellular adhesion.

The wettability property of electrospun mats is a crucial characteristic for the surface to cell interaction, once that cells may attach and proliferate better on hydrophilic mats surface. It is important to mention that a balance between hydrophobic and hydrophilic behavior is necessary. The first mats developed had hydrophobic behavior, showing WCA higher than 90°. In order to ameliorate the hydrophobicity of the mats, all scaffolds underwent eight seconds of plasma treatment. Then the contact angle was decreased from 130° to 32°, 136° to 31°, 135° to 36, 136° to 31°, 87° to 31, for neat PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI, respectively. This improved hydrophilic characteristic on the mats is beneficial for cell attachment.

Regarding the mechanical properties, the results show that PCL blended with LPEIs have better mechanical properties to the scaffolds than BPEIs scaffolds, which have almost the same mechanical properties as PCL. The blend of PEIs may reduce the tensile break of nanofibrous scaffolds. Mechanical stability of the scaffolds is desirable to provide cell growth and proliferation and degrade itself while the patient natural ECM starts regenerating the injured body sites.

The biocompatibility of the scaffolds was crucial for this study. First, we conduct cell-to-surface interaction study, which was made by SEM. The SEM images show the HDFs dispersion and morphology within the fibrous mats observed by SEM, by day 7, cells covered the 3D porous structure surface. HDFs on PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI expressed good cell-cell contact with almost no exposed fibers, the improved biocompatibility and hydrophilicity of the electrospun mats after plasma treatment, as previously mentioned, act to promote proliferation and migration of the cells. This attachment behavior plays a major role in skin tissue engineering and wound healing process as HDFs need strong cell ECM interaction and good cell-cell contact. This was followed by HDFs cell viability performed by MTS assay. We followed up the HDFs viability for ten days, from the data obtained we could conclude that none of the mats developed had toxicity effect to HDFs cells. The CMFDA assay, helped us to obtain semiquantitative information about living and dead cells. All the electrospun mats displayed no discernible cytotoxicity to HDF cells, therefore, confirming the biocompatibility of all scaffolds for HDF. Interestingly, the scaffold PCL_5LPEI has shown a decreased HDF proliferation, although, no cytotoxicity is presented. Last but not least assay to assure collagen expression for the mats developed was the Sirius Red collagen. Data showed high intensity of staining materials was observed on PCL_2LPEI, PCL_5LPEI, PCL_2BPEI electrospun scaffolds displaying an overall enhancing effect in collagen expression in HDFs cells and proving these scaffolds as an indispensable method for the fabrication of superior nanofibrous scaffolds for skin tissue engineering.

The evaluation of cytotoxicity for B16 murine melanoma cells was performed by SEM and MTS assay. The SEM images show B16 cells dispersion and morphology after 24 hours of incubation time. The cells had good adhesion behavior, this is an important characteristic since that cell attachment is required for drug delivery system. On the mats PCL, PCL_2LPEI and PCL_2BPEI the B16 cells do not present the wounded morphology of health B16 melanoma cells, it is possible to observe that the cells are flatted and spread out along the fibers. In PCL_5BPEI SEM image, is clearly noted the degradation of the cell membrane, although still attached to the surface of the electrospun mats. The Melanoma cell viability was monitored after 24 hours of incubation time. When compared to TCP, the mats PCL, PCL_2LPEI,

PCL_5LPEI, presented good cell viability, which is the contrary of what we proposed to do as an alternative for anticancer melanoma approach. On the other hand, the mats PCL_2BPEI and PCL_5BPEI had the higher anticancer activity when compared to our positive control Doxorubicin.

Another conducted study to gain insights of cell morphology was the confocal image. The first study conducted was for the melanoma cells, 24 hours after seeding the cells. The data showed multiple layers for TCP, the cells had a normal proliferation and growth, however, we had a different outcome for PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI, the images suggest cytotoxicity for the cells, the membrane becomes rounded, which indicates cell death. The major morphological differentiation was observed in PCL_5LPEI, PCL_2BPEI, PCL_5BPEI. Interesting, in our study, we did not use any green stain, which was observed on the layer of the scaffolds for PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, and PCL_5BPEI, this suggests that the melanin produced by melanoma cells give green fluorescent color, this is not observed in TCP, which served as control. Next, we conducted the same study for HDFs, we gained insights of the nucleus and cells membrane. The data suggest nontoxicity presented in the mats, due to the normal proliferation rate and healthy morphological shape of cells membrane and nucleus of HDFs seeded in PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, and PCL_5BPEI.

In conclusion, to mimicking the formation of ECM, which regulates and supports cell activities, we developed nanofibrous scaffolds based on PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, and PCL_5BPEI for the use of human dermal fibroblast during the early stage for skin tissue regeneration; this approach may be useful for injured skin as burns, diabetic foot ulcers, chronic wounds and to accelerate the wound healing process. Surface chemical characterization and morphological analysis confirmed the successful formation of PCL and PCL blended with PEIs composite structures. The surface wettability issue was overcome by plasma treatment. Biological studies with HDFs shown no toxicity for all mats, do not affect the surrounding tissue, it possesses good cell adhesion, cell proliferation and collagen release properties. The porous nanofibrous scaffolds interconnection may provide more structural space for HDFs adhesion and proliferation, permitting efficient metabolic wastes and nutrients exchanges. Together these data demonstrate that PCL blended with PEIs nanofibrous mats can provide multifunctional scaffolds properties, ranging from collagen secretion, cell adhesion and proliferation, acting as localized delivery of biomolecules to the injured skin, possessing the possibility to accelerate wound healing, useful for skin tissue engineering applications. Regarding the B16 murine melanoma cells, we could conclude that PCL_2BPEI and PCL_5BPEI had the higher anticancer effect, it is important to note that PCL_2BPEI had better cell viability for HDFs, compared to PCL_5BPEI. Protein kinase (PKs) plays an important role in

regulating various cellular functions, so, dysregulation of specific PKs activity is closely associated with many diseases, including cancer. As mentioned previously, there are various PKs, among them, the PKCα is reported as a suitable marker to distinguish carcinogenic cell from healthy cells, once that in tumour cells the PKCα activity is much higher than in healthy cells. The buffering capacity of the polyethyleneimine main chain results in polyplexes efficient escape from the endosome, showing a clear-cut response towards PKs activity. Together, all these data suggested that PCL_2BPEI composite nanofibrous mats would provide multifunctional scaffolds properties for possible skin engineering, wound healing system and anti-carcinogenic drug deliver application for melanoma skin cancer.

5.2 Future Research Guidelines

Upon completion of this doctoral thesis, it is likely to think that we could improve and add some scientific knowledge to this related field. Notwithstanding, it is also a very common feeling that although we have conducted an important and relevant work, it is not fully finished and it possesses a lot of new possibilities and alternatives to enrich and explore. Bearing this idea, it is true to believe that this presented work can be thought as an initial matrix and start point for further investigations, which might contribute a better understanding and promoter better insights to the scientific community. Future work in this topic may lead to wider and encompass other nanotechnological and biofunctionalization of electrospun mats to accelerate wound healing process, promote skin reconstruction, enhance drug delivery copolymers to the target carcinogenic cells, hoping in the near future to delivery this idea to the market, providing better the patient´s perspective of life, health and wellbeing, minimizing the suffering and side effect commonly reported in patient´s under traditional cancer approaches such as chemo and radiotherapy.

Thus, in order to complement and enhance the present doctoral investigation, we intend and propose in the near future to develop new experimental and practical framework, developing a supplementary set of trial aiming at:

- Investigate the developed electrospun mats and its cell viability to melanocytes and keratinocytes cells;
- Promote antimicrobial test in order to evaluate their effectiveness in bacterial strains, especially in Staphylococcus aureus, MRSA, *Klebsiella pneumonia*;
- Develop a scaffold that could mimic the skin surface for the clinical trial, containing keratinocytes, melanocytes, human dermal fibroblast and melanoma cells;

- Try new technique as 3D printing technology for the development of new biofunctional materials;
- Perform *in vivo* test, to gain better insights of the developed electrospun scaffolds and their interactions to body;
- If possible, in the future, to develop clinical trial in hospital facilities where the scaffolds would be put under real circumstance and conditions, aiming to ascertain the new alternative approach for the patient´s diagnosed with melanoma;
- Study the economic and health impact of the scaffolds in the medical environment.

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