

Electrospun Linear and Branched Nanofibrous Scaffolds for Potential Therapeutic Application in Melanoma

Lucas B. Naves^{1,2,3}
Chetna Dhand^{3,4}
Seow Theng Ong⁵
Chinnasamy Gandhimathi³
Jayarama Reddy Venugopal³
Luis Almeida²
Navin k. Verma^{4,5}
Rajamani Lakshminarayanan⁵
Seeram Ramakrishna^{3,6}

¹CAPES Foundation, Ministry of Education of Brazil, Brazil
²Centre for Textile Science and Technology, University of Minho, Portugal
³Department of Mechanical Engineering, Center for Nanofibers & Nanotechnology, National University of Singapore, Singapore
⁴Anti-Infectives Research Group, Singapore Eye Research Institute, Singapore
⁵Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore
⁶Guangdong-Hongkong-Macau Institute of CNS Regeneration (GHMICR), Jinan University, China

Abstract

In this study, we present a potential alternate 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 74 ± 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 to determinate the biocompatibility with all cells, and confocal images to give as 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_BPEI nanofibers. 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, therefore it can be an emerging technology for skin regeneration, wound healing process and treatment of melanoma through electrospun drugs delivery system.

Key words

Melanoma; Scaffolds; Electrospinning; Drug Delivery; Tissue Engineering

Introduction

Nanofibers are widely used in various medical biomedical application such as tissue engineering, cell therapy, regenerative medicine, drug delivery and cancer therapy. These fibers have 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, the scaffolds properties, they have been 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 provides abundant binding and adhesion to cell receptors. The strong cell- matrix interaction allows to develop cell engineering, organs and tissues [1]. 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 [2].

Several studies have been carried out on nanofibers composites, demonstrating that for biomedical and biological application, nanofibers composites are better option than their monophasic nanofibers [3].

Article Information

DOI: 10.31021/jcro.20181113
Article Type: Research Article
Journal Type: Open Access
Volume: 1 **Issue:** 3
Manuscript ID: JCRO-1-113
Publisher: Boffin Access Limited

Received Date: 16 July, 2018
Accepted Date: 29 August, 2018
Published Date: 31 August, 2018

*Corresponding author:

Luis Almeida
Centre for Textile Science and Technology
University of Minho, Braga
4704553, Portugal
Tel: 351-96305727
E-mail: lalmeida@det.uminho.pt

Citation: Naves LB, Dhand C, Ong ST, Gandhimathi C, Venugopal JR, et al. Electrospun Linear and Branched Nanofibrous Scaffolds for Potential Therapeutic Application in Melanoma. J Cancer Res Oncobiol. 2018 Aug;1(3):113

Copyright: © 2018 Naves LB, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 international License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

The interaction within 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 an unique properties [2].

In the 21st century, drugs microencapsulation technology has been applied for drugs deliveries. 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 when designing new pharmaceutical agents; these agents must be identified and specified before the drug design. Ethical approval of pharmaceutical products should be obtained to demonstrate use of the drugs, as being supported by results of clinical trials and animals experiments. Lucas et. al. [4], 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 technical issue should be considered when the pharmaceutical agents microencapsulated technology is employed for mass production. When these drugs are designed for therapeutic application, is very important to identify and examine careful the [4] chemical, physical and therapeutic properties of microcapsules. To avoid an overdose administration of 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 [5].

Drugs topical delivery is related to the application of drugs to the skin surface, aiming to delivery therapeutic agents trough skin into the systemic circulation or blood stream (transdermal delivery) or to pathological sites of the skin (dermal delivery) [6]. As example of skin disorder treated using transdermal delivery we can mention severe pain which is treated with fentanyl [7], while eczema and psoriasis are treated using dermal delivery [8]. The efficacy of topical drugs delivery is directly related to the diffusion of therapeutic agent through the skin and the drug formulation.

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 [9,10]. Therefore (PEI) has been used to transfect a variety of cell-types, both *in vitro* and *in vivo* [11]. 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 [12-14]. 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 are 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) [12]. Scientists have done several approaches for melanoma cancer treatment, and gene therapy including both PEI polymers, either Linear or branched [15], given by their attractive properties for drugs delivery systems [16]. 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 scape and well nuclease digestion [17]. The kinetics of cells association of both, is very important, once that, there is substantial internalization of PEI bound to the plasma membrane of malignance 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 [18]. The toxicities and transfection efficiencies of PEIs differ in structure dependent or mass dependent manner, so far is very difficult to have any defined relationship between these parameters [19,20]. The use of PEIs might be limited by its proinflammatory effects and high toxicity effects [21].

Using electrospun technology possible to develop new methods in tissue engineering, as organs and tissue that are developed by using a combination of biocompatible polymer scaffolds and patient's own cells.

Up to now, scientists have focused their studies on developing polymer scaffolds using biocompatible and biodegradable polymers, designing a scaffold that can mimic the extracellular matrix (ECM) proteins, regulating the cells activities and providing mechanical support [22]. Skin substitutes such as autografts, allografts and xenografts have been used for wound healing of patients with significant dermal injuries [23].

Some studies showed that the cell adhesion and proliferation can be enhanced by protein absorption on the surface of the scaffolds [24,25]. For this study we used as main polymer polycaprolactone (PCL) which is a synthetic polymer, semi- crystalline, extensively used for biomedical applications due to its properties like high tensile strength, low melting point, biodegradability and non-toxic nature [26]. Skin tissue engineering, PCL has been investigated as potential matrix for the regeneration of damaged tissues. However, PCL use is limited due to its lack of functional groups, high hydrophobicity, neutral charge and poor bioregulatory activity [27]. To overcome these limitations various post-processing and physico-chemical surface modification techniques have been reported in the literature [28]. It is possible to bring down the disadvantages of PCL by blending another polymer with desired characteristics [29].

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 [30]. In addition to that, chemotherapy treatment is very invasive for patients having low self-immune defense. This is a complex disease that arises through multiple etiologic pathway. Limited number of treatments are available for melanoma medical application, mostly of patients with a more aggressive form of the disease with neither long-lasting nor effective treatment presently exists decline treatment [31]. 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 1/2 proteins, involved in the control of cell proliferation, growth and migration [32]. Mutations in this pathway may play a major role in the progression and development of melanoma cancer [33]. The National Comprehensive Cancer Network (NCCN) guidelines metastatic melanoma can be treated with dacarbazine (DTIC) which is FDA drug approved [34], also with paclitaxel, temozolomide; these treatments are not specific MEK and BRAF inhibitors [35]. Several studies have been carried out on nanofibers composites, demonstrating that for biomedical and biological application, nanofibers composites a better option than their monophasic nanofibers. When compared chitosan nanofibers to blended polycaprolactone (PCL)/chitosan, the cell proliferation increased (> 50%) and obtained better mechanical properties to the composite nanofibers polycaprolactone (PCL)/chitosan than the monophasic chitosan nanofibers system [3].

Therefore, in this article we investigated the cytotoxicity of PEIs (LPEI/BPEI) electrospun mats blended with PCL against a panel of cells, which includes melanoma (B16) and humam dermal fibroblast (HDF). We developed five difeerent mats with differnte LPEI and BPEI loads, namely PCL, blended PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI. Using MTT assay we have shown the high

cytotoxicity of PCL coated with PEIs for melanoma cells. Finally, we demonstrate, using SEM microscopy, CMFDA, and Sirius red collagen, that crosslink of LPEI/BPEI on to an (PCL) matrix can attenuate the cytotoxicity for HDFs. It was noted that after 6 days, the collagen release profile was increased. The present study suggests that the mats developed may increase the skin regeneration process and at the same time promote apoptosis of melanoma cells, therefore it can be an emerging technology for skin regeneration, wound healing process and treatment of melanoma through electrospun nanofibers for drugs delivery applications

Materials and Methods

Materials

Linear (Mn~20,000, catalogue no. 764965, Sigma) and branched (Mn ~10,000, catalogue no. 408727, Sigma) polyethylene imine hydrochloride, Polycaprolactone, 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 primary dermal fibroblast culture was from American Type Culture Collection (Manassas, VA, USA).

Electrospinning of polycaprolactone and LPEI and BPEI polymers

Five different types of 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), were developed. All the mats were based on 10% w/v of PCL, which was prepared from methanol and chloroform (3:7) solution, left overnight stirring 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 solution left over night was 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) For simplicity, the mats were labeled as follows: PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI.

Morphological characterization by Scanning Electron Microscopy (SEM)

The scanning electron microscopy analysis of all mats was performed on a FE-SEM (FEI-QUANTA 200F, The Netherlands), accelerate voltage of 15 kV after sputter coating the samples with gold. The Images Analysis Software, have been used to estimate the average diameter for various scaffolds. The diameter sample of each sample, ~100 nanofibers diameter 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 hexamethyl disilazane (HMDS), left overnight in the fume hood. Each sample was coated with gold, and analyzed under SEM.

Determination of mechanical properties

The determination of mechanical parameters such as: Work of Failure, Failure Strain, Young's Modulus and Tensile Strength, were done following the protocol ASTM D882-02 (Standard Test Method for Tensile Properties of Thin Plastic Sheet), for all the electrospun mats. To carry out the experiments was used, at ambient conditions, a tabletop tensile tester (Instron 5345, USA) using a capacity of load cell of 10 N. 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 cross- head speed of 5 mm min⁻¹. The results reported are the average from 3 independent measurements.

Determination of 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 mats surface. 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.

Human Dermal Fibroblasts (HDF) and Melanoma (B16) cancer cell culture

HDF and B16 cells were cultured in DMEM supplemented with 10% of FBS and antibiotics. We used 75 cm² cell culture flasks for culturing both the cells. The cells were incubated at 37°C in humidifier CO₂ incubator for 1 week and fed with fresh complete DMEM every 3 days. It was used trypsin-EDTA and the cells were re-plated 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 stainless steel rings. 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 density of 2x10⁴ cells well⁻¹ on PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI scaffolds. Tissue culture plate (TCP) served as positive controls.

Sirius red for collagen assay

The fibrous scaffolds were collected on 15mm cover slips, 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 cover slips. 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 solvents. The scaffolds were finally soaked with complete media overnight. The HDF cells were seeded at the density of 1 x 10⁴ cells well⁻¹. The plates were kept into the incubator at 37°C in humidified CO₂ 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 red 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 into the incubator for one hour. The wells were finally washed with 100% ethanol and observed under the Leica microscope for collagen release profile. In Figure 1 we present an overall summary of this study. While figure 3 showing the overall summary of the study made for HDFs and B16 melanoma cells.

Statistical analysis

Experiments were conducted 5 times and all the data presented were expressed as mean ± standard deviation (SD). Statistical analysis was plotted using Student's *t*-test; *p* ≤ 0.001 was considered to be highly significant.

Analysis and Interpretation of Results

Morphological analysis of electrospun nanofibrous scaffolds

Table 1 shows the diameters and the water contact angles of the five different types of nanofibers (Figure 2). Notably, we observed

Nanofibrous construct	Fiber diameter (nm)	Water contact angle (°)	Young's modulus (MPa)	Failure Strain (%)
PCL	331 ± 74	32	7.2 ± 2.0	135.8
PCL_2LPEI	261 ± 79	31	15.57 ± 6.8	79.2
PCL_5LPEI	258 ± 109	36,6	15.18 ± 3.97	82.4
PCL_2BPEI	271 ± 81	31,1	5.97 ± 3.27	98.3
PCL_5BPEI	419 ± 233	32,5	7.53 ± 3.96	87.3

Table 1: Characterization of biocomposite nanofibrous scaffolds

an increase in the fibre diameter in 10% of poly-(ϵ -caprolactone) blended with 5% of branched polyethylenimine scaffolds, which could be due to possible decrease in the conductivity of the polymer solution.

All the scaffolds prepared in this study showed hydrophobic behavior with a contact angle higher than 87° , as we can observe in Figure 3 for non-treated mats. To ameliorate the hydrophilic properties, the scaffolds kept for plasma treatment for 8s. The observed results after plasma treatment are shown in Figure 3. After plasma treatment, we could observe the improvement of hydrophilic properties. The hydrophilic properties of the nanofibrous scaffolds is directly proportional to the rate of water absorbance. This is showed in Table 1, where we can observe the water contact angle values dropped, ranging from 31° to 37° .

Characterization of nanofibers by FT-IR

FTIR analysis was used to detect the possible changes in the chemical structure of the electrospun materials (Figure 4). 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 [36]. The spectra shows characteristic absorption peaks of PEI at 1580 and 1460 cm^{-1} [37], in the linear PEI, the amide group I peak is at 1648 cm^{-1} , and 1552 cm^{-1} corresponds with the amide II peaks [38]. The branched PEI shows its characteristic absorption bands at about 3440 and 1654 cm^{-1} assigned to the stretching vibration of the group -NH_2 [38].

Mechanical properties

The mechanical properties of PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI nanofibrous scaffolds are shown in Table 1, and the scaffolds revealed a characteristic semi linear stress-strain curve. The Young's modulus obtained for PCL, PCL_2LPEI, PCL_5LPEI,

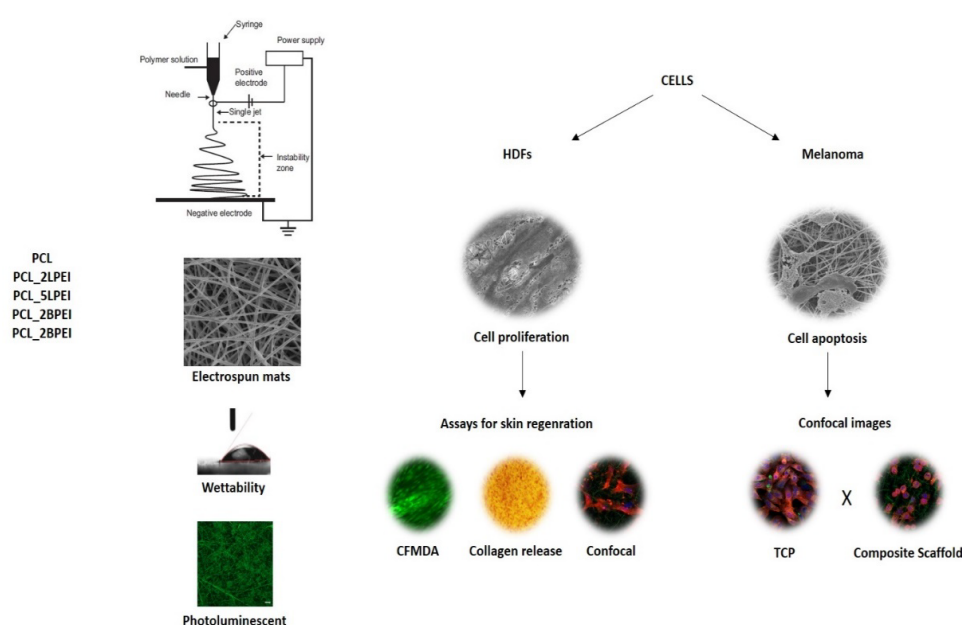


Figure 1: Overall summary of the present work

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.

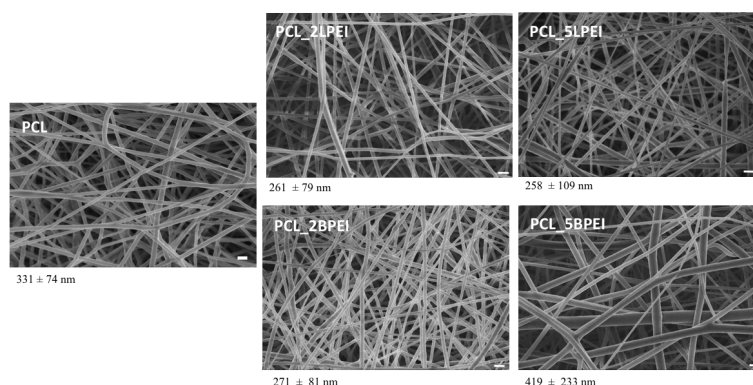


Figure 2: Scanning Electron Microscope (SEM) images of all nanofibers (500x magnification). Scale bar = $1\mu\text{m}$

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, was higher when compared to the PCL, and lower for PCL_5BPEI and PCL_2BPEI. The observed results proved the increasing 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. Crosslink of PEIs may reduce the tensile break of nanofibrous scaffolds observed in Figure 5. 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.

Interaction of cells and scaffolds

The cell morphology and biomaterials interactions (cell migration, proliferation and differentiation) have been studied intensively as they can provide the key to factors for the clinical success of an implanted device and tissue repair [39]. The interaction between the nanofibrous scaffolds and cells depends on the chemical and physical properties of the biomaterials [40]. The SEM images (Figure 6 and 7) showed normal cell morphology and adhesion on PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI and TCP scaffolds on day 4 and 7. We observed higher HDFs proliferation on PCL_5LPEI and PCL_2BPEI. All the mats improved cell proliferation compared from day 4 to day 7 [39,41,42].

In Figure 8, is possible to observe the MTT assay, which the results emphasize the good biocompatibility of all mats developed, PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI, PCL_5BPEI, and TCP that served as control. In general, MTT assay is based on the fact that metabolic active cells interact with a tetrazolium salt (in a MTT reagent), therefore, producing a soluble formazan dye. The absorbance intensity is an indication of viable cells present, the higher the absorbance, the higher the viability is [42]. To obtain better insight, data was expressed in cell proliferation ratio after dividing cell number at each time point with initial cell density, the assay was performed on day 1, 4, 7 and 10 after seeding the HDFs cells into the scaffolds. The relatively high optical densities after 4 days of incubation, indicated that PCL, LPEI and BPEI supported cell adhesion on nanofibrous matrices [39]. Results indicated that there was no cytotoxicity rate to all mats. The scaffolds volume ratio and surface area increased the nutrients and oxygen transport, therefore promoting good cell adhesion, growth and proliferation for all the scaffolds. It was not observed apoptotic behavior of human dermal fibroblast cells, on the contrary, over the course of 10 days, the proliferation rate increased meaning a good biocompatibility for HDF, which may be useful for wound healing.

We next determined cell viability using CMFDA live-dead staining. After four days post seeding (p.s.) of HDF cells on various scaffolds, the cells were stained with green CMFDA dye (5-Chloromethylfluorescein diacetate) to obtain semi qualitative 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 (Figure 9).

Next, we determined secretion of collagen using Picro-Sirius red (Figure 10). After 6 days, PCL_2BPEI, PCL_5BPEI and PCL_2LPEI showed higher collagen release than that in control.

The nanofiber mats show anticancer activity on melanoma cells

When melanoma cells were cultured on fiber mats for 24 h, a reduction of cells density and altered cellular morphologies were observed on was observed the mats PCL_2BPEI and PCL_5BPEI, when compared to TCP (Figure 11).

The metabolic assay for living cells MTT, was also performed to melanoma cells, as we can observe in Figure 12. The reading was taken after 24 hours of incubation time, data obtained suggested high toxicity for the mats PCL_2BPEI and PCL_5BPEI, doxorubicin is an anticancer drug and was used as positive control. TCP served as negative control.

To gain insights of cell morphology, confocal images of the melanoma cells cultured on various scaffolds are shown in Figure 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 suggest 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 cultured media.

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 14). As comparison on the right 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.

Protein kinase C (PKC) family of serine/threonine was one of the first protein kinase to be identified. It is a heterogeneous group of

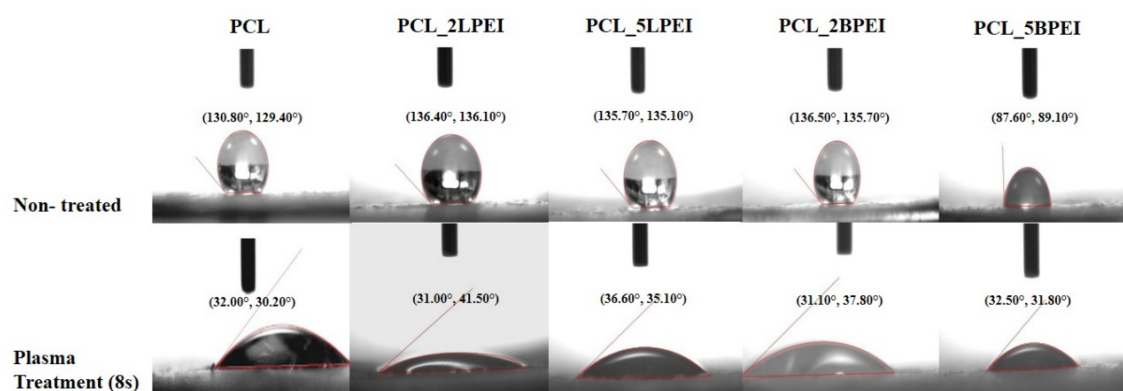


Figure 3: Water contact angle for all mats, non- treated mats on the left and after 8s of plasma treatment on the right

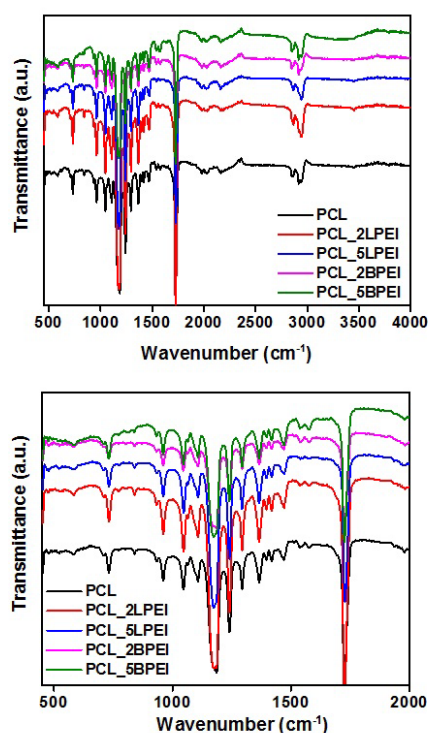


Figure 4: Fourier transform infrared spectroscopy spectra for all nanofibrous mats

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.

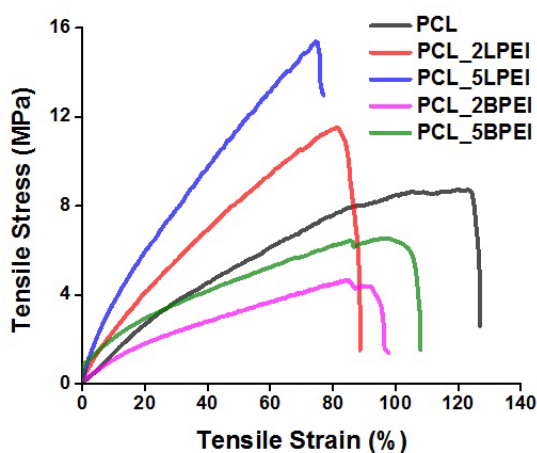


Figure 5: Tensile stress-strain curves of PCL, PCL_2LPEI, PCL_5LPEI, PCL_2BPEI and PCL_5BPEI

Abbreviations: PCL, -poly-(ϵ -caprolactone); PCL_2LPEI, poly-(ϵ -caprolactone) blended with 2% of linear 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.

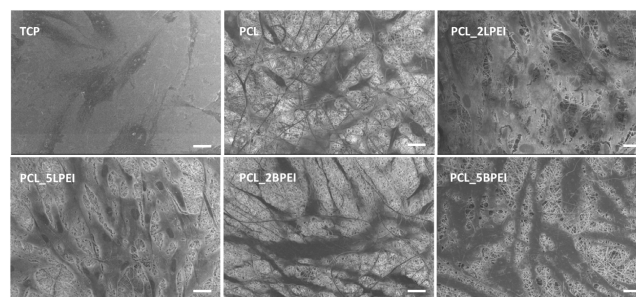


Figure 6: Scanning Electron Microscope (SEM) images show the nanofibers-cell interaction on the day 1.

PCL, 2LPEI, 5LPEI, 2BPEI, 5BPEI and TCP (500x magnification) on day 7. Scale bar = 1 μ m

Abbreviations: PCL, -poly-(ϵ -caprolactone); 2LPEI, poly-(ϵ -caprolactone) blended with 2% of linear polyethylenimine; 5LPEI, poly-(ϵ -caprolactone) blended with 5% of linear polyethylenimine; 2BPEI, poly-(ϵ -caprolactone) blended with 2% of branched polyethylenimine; 5BPEI, poly-(ϵ -caprolactone) blended with 5% of branched polyethylenimine.

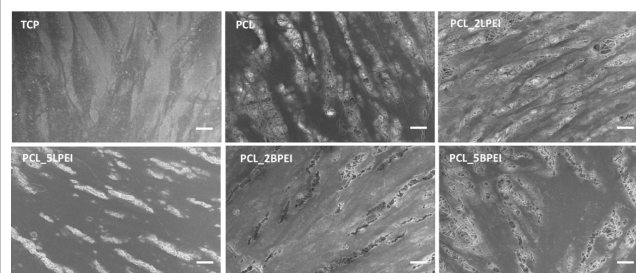


Figure 7: Scanning Electron Microscope (SEM) images show the nanofibers-cell interaction on the day 6. PCL, 2LPEI, 5LPEI, 2BPEI, 5BPEI and TCP (500x magnification) on day 7. Scale bar = 1 μ m.

Abbreviations: PCL, -poly-(ϵ -caprolactone); 2LPEI, poly-(ϵ -caprolactone) blended with 2% of linear polyethylenimine; 5LPEI, poly-(ϵ -caprolactone) blended with 5% of linear polyethylenimine; 2BPEI, poly-(ϵ -caprolactone) blended with 2% of branched polyethylenimine; 5BPEI, poly-(ϵ -caprolactone) blended with 5% of branched polyethylenimine.

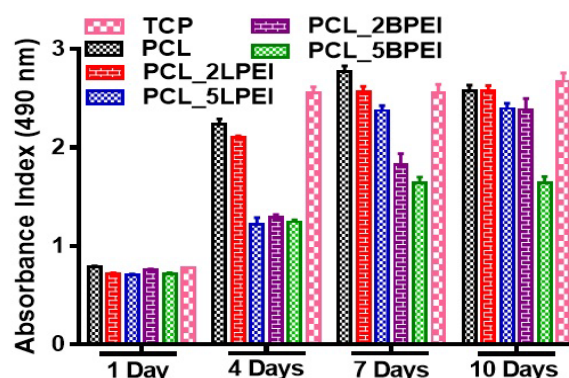


Figure 8: MTS assay of electrospun mats after 1, 4, 7 and 10 days of incubation time of human dermal fibroblast (HDF) cells. Absorbance Index 490 nm.

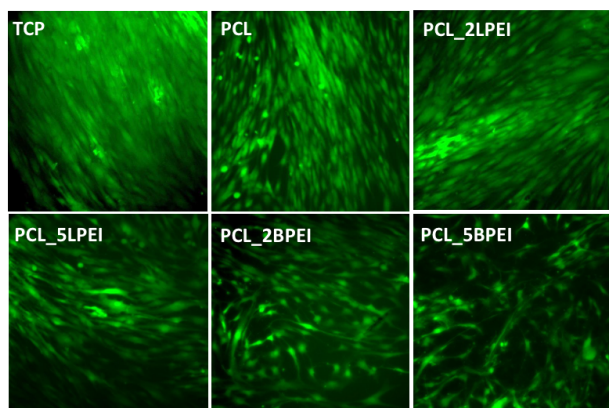


Figure 9: CMFDA confocal images of HDF cultured on TCP, PCL 10%, PCL 10%_LPEI2%, PCL 10%_LPEI 5%, PCL 10%_BPEI 2%, PCL 10%_BPEI 5%. Scale bar = 20 μ m. **Abbreviations:** PCL, -poly-(ϵ -caprolactone); PCL_2LPEI, poly-(ϵ -caprolactone) blended with 2% of linear polyethylenimine; PCL_5LPEI, poly-(ϵ -caprolactone) 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.

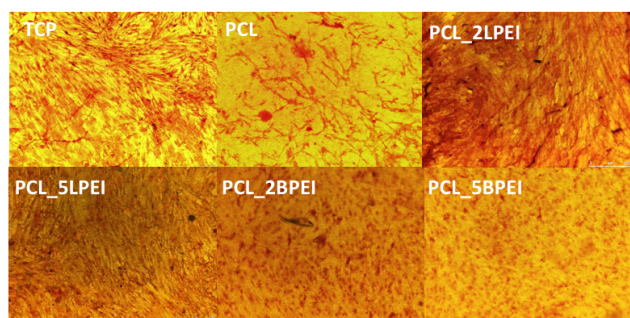


Figure 10: Sirius red collagen assay, observation of collagen release on the day 6 for cultured HDFs

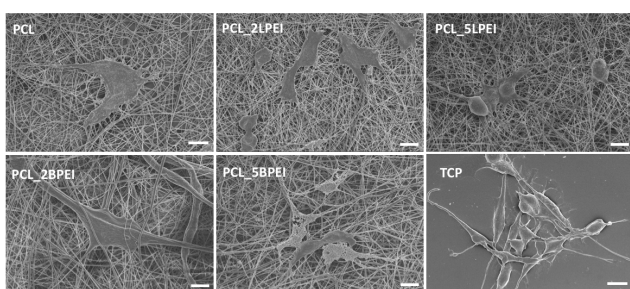


Figure 11: Scanning Electron Microscope (SEM) images show the biomaterial-cell interaction with murine melanoma cells B16 after 24 hours of incubation time.

enzymes integrating and receiving signals in both normal melanocytes and melanoma pathology. The chromatographic purification of PKC shows that this activity was composed by at least three distinct species, designated as α , β and γ isotypes [43]. Nowadays, it is known that PKC isotypes of mammals 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 provide insights and defining a number of regulatory elements in PKC which confer to each isotype specific activation signals and location [44].

The alteration of PKC enzyme activation and expression contribute directly to the malignant phenotype of melanoma in both tumour suppressive and oncogenic roles. These enzymes are involved in a varied array of biological process including, migration, cell polarity, proliferation, differentiation and apoptosis. PCK enzymes have profound influences on actin cytoskeleton organization and PCK α , which have been linked to melanoma invasion. Thus, activation and/or alteration in the expression of PCK α cooperate with β 3 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 β 3 is associated with melanoma invasion and progression [45].

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 [46].

Agonist is a chemical that binds to a receptor and activate the receptor to produce biological response. PCK 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 [45].

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

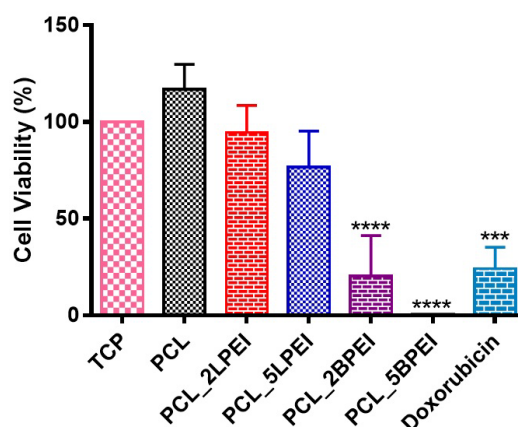


Figure 12: MTS assay reading of electrospun mats after 24 hours of incubation time of murine melanoma cells B16. (****, and *** represents high cytotoxicity). Absorbance Index 490 nm

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.

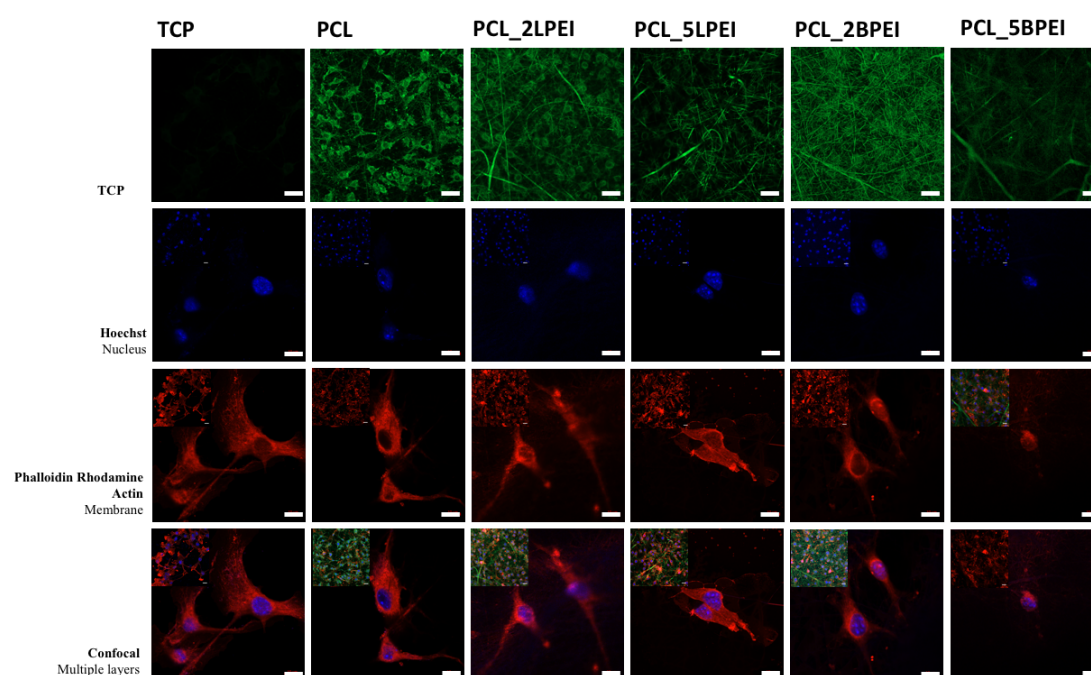


Figure 13: Confocal images of electrospun mats seeded with murine melanoma cells B16 after 24 hours of incubation time.

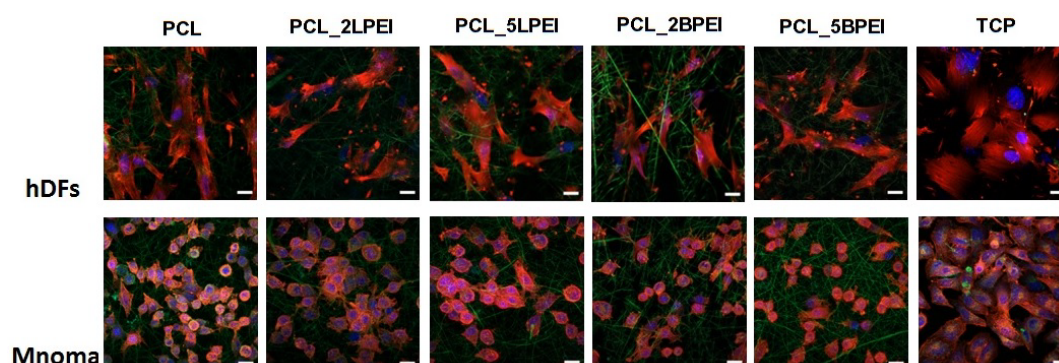


Figure 14: Confocal images of electrospun mats seeded with human dermal fibroblasts (HDFs) and murine melanoma cells B16 (Mnoma) after 24 hours of incubation time

Abbreviations: PCL, -poly-(ϵ -caprolactone); PCL_2LPEI, poly-(ϵ -caprolactone) blended with 2% of linear 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

As LPEI and BPEI can target PKC α 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 14.

Conclusions

Mimicking the formation of ECM, which regulates and supports cell activities, we developed nanofibrous scaffolds based on PCL 10%, PCL 10%_LPEI2%, PCL 10%_LPEI 5%, PCL 10%_BPEI 2%, PCL 10%-BPEI 5% 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 and chronic wounds. 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, 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_5BPEI was toxic for HDFs cells. Protein kinase (PKs) play an important role in regulating various cellular functions, so, dysregulation of specific PKs activity is closely associated with many disease, including cancer. As mentioned previously, there are various PKs, among them, the PKC α is reported as suitable marker to distinguish carcinogenic cell from healthy

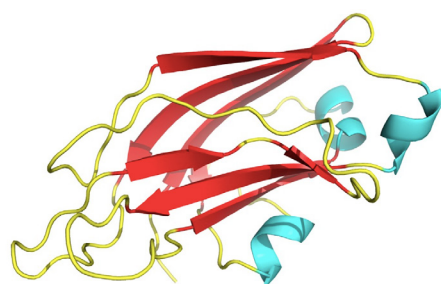


Figure 15: Graphical representation of Protein kinase C, α subunit

cells, once that in tumour cells the PCK α activity is much higher than in healthy cells. The buffering capacity of the polyethylenimine main chain, results in polyplexes efficient escape from 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 drug delivery application (Figure 15).

Acknowledgment

Authors wish to acknowledge financial support from CAPES foundation for the Ph.D. Grant with process Number 13543/13-0, Brazilian Ministry of Education-Brazil. Authors also like to thank FEDER funds through the Competitiveness Operational Programme—COMPETE and national funds through FCT—Foundation for Science and Technology (POCI-01-0145-FEDER-007136). RL thanks the funding support from Singapore National Research Foundation under its Translational and Clinical Research Flagship Programme (NMRC/TCR/008-SERI/2013) and administered by the Singapore Ministry of Health's National Medical Research Council and Co-operative Basic Research Grant from the Singapore National Medical Research Council (Project No. NMRC/CBRG/0048/2013). NKV acknowledges Start-Up Grant, Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore.

References

1. Yang F, Murugan R, Wang S, Ramakrishna S. Electrospinning of nano/micro scale poly(l-lactic acid) aligned fibers and their potential in neural tissue engineering. *Biomaterials*. 2005 May;26(15):2603-2610.
2. Ramalingam S, Ramakrishna S. Nanofiber Composites for Biomedical Applications: Introduction to nanofiber composites. 1st ed. Ramalingam S, Ramakrishna, Ed. India (Chennai): Elsevier Woodhead Publishing; 2017.pp. 3-29.
3. Prabhakaran MP, Venugopal JR, Chyan TT, Hai LB, Chan CK, et al. Electrospun biocomposite nanofibrous scaffolds for neural tissue engineering. *Tissue Eng Part A*. 2008 Nov;14(11):1787-1797.
4. Naves LB, Dhand C, Almeida L, Rajamani L, Ramakrishna S. *In vitro* skin models and tissue engineering protocols for skin graft applications. *Essays Biochem*. 2016;60(4):357-369.
5. Orive G, Hernández RM, Rodríguez Gascón A, Calafiore R, Chang TM. History, challenges and perspectives of cell microencapsulation. *Trends Biotechnol*. 2004;22(2):87-92.
6. Garvie-Cook H. Topical Drug Delivery, in *Novel (Trans) dermal Drug Delivery Strategies*. Switzerland: Springer International Publishing ; 2016. pp. 5-28.
7. Delgado-Charro MB, Guy RH. Effective use of transdermal drug delivery in children. *Adv Drug Deliv Rev*. 2014 Jun;73:63-82.
8. Flowers FP. Transdermal and Topical Drug Delivery: From Theory to Clinical Practice. *Annals of Pharmacotherapy*. 2004;38:726-727.
9. Zhou Z, Murdoch WJ, Shen Y. A linear polyethylenimine (LPEI) drug conjugate with reversible charge to overcome multidrug resistance in cancer cell. *Polymer*. 2015 Oct;76(12):150-158.
10. Huh SH, Do HJ, Lim HY, Kim DK, Choi SJ, et al. Optimization of 25 kDa linear polyethylenimine for efficient gene delivery. *Biologicals*. 2007 Jun;35(3):165-171.
11. Reed SE, Staley EM, Mayginnis JP, Pintel DJ, Tullis GE. Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. *J. Virol. Methods*. 2006 Dec;138(1-2):85-98.
12. Fox SJ, Fazil MH, Dhand C, Venkatesh M, Goh ET, et al. Insight into membrane selectivity of linear and branched polyethylenimines and their potential as biocides for advanced wound dressings. *Acta Biomater*. 2016 Jun;37:155-164.
13. Parhamifar L, Larsen KA, Hunter, Andresen TL, Moghimi SM. Polycation cytotoxicity: a delicate matter for nucleic acid therapy—focus on polyethylenimine. *Soft Matter*. 2010;6(17):4001.
14. Spoden GA, Besold K, Krauter S, Plachter B, Hanik N, et al. Polyethylenimine is a strong inhibitor of human papillomavirus and cytomegalovirus infection. *Antimicrob Agents Chemother*. 2012 Jan;56(1):75-82.
15. Ogris M, Walker G, Blessing T, Kircheis R, Wolschek M, et al. Tumor-targeted gene therapy: Strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. *J Control Release*. 2003 Aug 28;91(1-2):173-181.
16. Park SC, Nam JP, Kim YM, Kim JH, Nah JW, et al. Branched polyethylenimine-grafted-carboxymethyl chitosan copolymer enhances the delivery of pDNA or siRNA *in vitro* and *in vivo*. *Int J Nanomedicine*. 2013;8:3663-3677.
17. Benjaminsen RV, Matthebjerg MA, Henriksen JR, Moghimi SM, Andresen T. The Possible 'Proton Sponge' Effect of Polyethylenimine (PEI) Does Not Include Change in Lysosomal pH. *Mol Ther*. 2013 Jan;21(1):149-157.
18. Seib FP, Jones AT, Duncan R. Comparison of the endocytic properties of linear and branched PEIs, and cationic PAMAM dendrimers in B16f10 melanoma cell. *J Control Release*. 2007 Feb 26;117(3):291-300.
19. Han HD, Byeon Y, Jeon HN, Shin BC. Enhanced localization of anticancer drug in tumor tissue using polyethylenimine-conjugated cationic liposomes. *Nanoscale Res Lett*. 2014 May 5;9(1):209.
20. Kwok A, Hart SL. Comparative structural and functional studies of nanoparticle formulations for DNA and siRNA delivery. *Nanomedicine*. 2011 Apr;7(2):210-219.
21. Kawakami S, Ito Y, Charoensit P, Yamashita F, Hashida M. Evaluation of proinflammatory cytokine production induced by linear and branched polyethylenimine/plasmid DNA complexes in mice. *J Pharmacol Exp Ther*. 2006 Jun;317(3):1382-1390.
22. Yoo CR, Yeo IS, Park KE, Park JH, Lee SJ, et al. Effect of chitin/silk fibroin nanofibrous bicomponent structures on interaction with human epidermal keratinocytes. *Int J Biol Macromol*. 2008 May 1;42(4):324-334.
23. Auger FA, Lacroix D, Germain L. Skin substitutes and wound healing. *Skin Pharmacol Physiol*. 2009;22(2):94-102.
24. Webster TJ, Ergun C, Doremus RH, Siegel RW, Bizios R. Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. *J Biomed Mater Res*. 2000 Sep 5;51(3):475-483.
25. Webster TJ, Siegel RW, Bizios R. Osteoblast adhesion on nanophase ceramics. *Biomaterials*. 1999 Jul;20(13):1221-1227.
26. Prabhakaran MP, Venugopal JR, Ramakrishna S. Mesenchymal stem cell differentiation to neuronal cells on electrospun nanofibrous substrates for nerve tissue engineering. *Biomaterials*. 2009 Oct;30(28):4996-5003.
27. Prasad T, Shabeena EA, Vinod D, Kumary TV, Anil Kumar PR. Characterization and *in vitro* evaluation of electrospun chitosan/

- polycaprolactone blend fibrous mat for skin tissue engineering. *J Mater Sci Mater Med*. 2015 Jan;26(1):5352.
28. Gupta D, Venugopal J, Prabhakaran MP, Dev VR, Low S, et al. Aligned and random nanofibrous substrate for the in vitro culture of Schwann cells for neural tissue engineering. *Acta Biomater*. 2009 Sep;5(7):2560-2569.
 29. Wang TJ, Wang JI, Lu JN, Young TH. Novel chitosan-polycaprolactone blends as potential scaffold and carrier for corneal endothelial transplantation. *Mol Vis*. 2012 Jan;18: 255-264.
 30. Zhang G, Liu S, Liu Y, Wang F, Ren J, et al. A novel cyclic pentapeptide, H-10, inhibits B16 cancer cell growth and induces cell apoptosis. *Oncol Lett*. 2014 Jul;8(1):248-252.
 31. Yang CH, Yue J, Sims M, Pfeffer LM. The Curcumin Analog EF24 Targets NF- κ B and miRNA-21, and Has Potent Anticancer Activity In Vitro and In Vivo. *PLoS One*. 2013 Aug 7;8(8):e71130.
 32. Colombino M, Capone M, Lissia A, Cossu A, Rubino C, et al. BRAF/NRAS mutation frequencies among primary tumors and metastases in patients with melanoma. *J Clin Oncol*. 2012 Jul;30(20):2522-2529.
 33. Casula M, Muggiano A, Cossu A, Budroni M, Caracò C, et al. Role of key-regulator genes in melanoma susceptibility and pathogenesis among patients from South Italy. *BMC Cancer*. 2009 Oct 3;9:352.
 34. Jilaveanu LB, Aziz SA, Kluger HM. Chemotherapy and biologic therapies for melanoma: do they work?, *Clinics in Dermatology*. *Clin Dermatol*. 2009 Nov-Dec;27(6):614-625.
 35. Coit DG, Andtbacka R, Anker CJ, Bichakjian CK, Carson WE 3rd, et al. Multiple Melanoma, Version 2 .2013 Featured Updates to the NCCN Guidelines. *J Natl Compr Canc Netw*. 2013 Apr;11(4):395-407.
 36. Elzein T, Nasser-Eddine M, Delaite C, Bistac S, Dumas P. FTIR study of polycaprolactone chain organization at interfaces. *J Colloid Interface Sci*. 2004 May;273(2):381-387.
 37. Zi Hou J, Xue HL, Li LL, Dou YL, Z, Wu ZN, et al. Fabrication and morphology study of electrospun cellulose acetate/polyethylenimine nanofiber. *Polym Bull*. 2016 Oct;73(10):2889-2906.
 38. Khanam D, Mikoryak C, Draper RK, and K. J. Balkus KJ, Electrospun linear polyethyleneimine scaffolds for cell growth. *Acta Biomater*. 2007 Nov;3(6):1050-1059.
 39. Gümüşderelioglu M, Dalkıranoglu S, Aydın RS, Cakmak S. A novel dermal substitute based on biofunctionalized electrospun PCL nanofibrous matrix. *J Biomed Mater Res A*. 2011 Sep 1;98(3):461-472.
 40. Guo X, Gough JE, Xiao P, Liu J, Shen Z. Fabrication of nanostructured hydroxyapatite and analysis of human osteoblastic cellular response. *J Biomed Mater Res A*. 2007 Sep;82(4):1022-1032.
 41. Venugopal JR, Zhang Y, Ramakrishna S. In Vitro Culture of Human Dermal Fibroblasts on Electrospun Polycaprolactone Collagen Nano fibrous Membrane. *Artif Organs*. 2006 Jun;30(6):440-446.
 42. Sadeghi AR, Nokhasteh S, Molavi AM, Khorsand-Ghayeni M, Naderi-Meshkin H, et al. Surface modification of electrospun PLGA scaffold with collagen for bioengineered skin substitutes, *Mate Sci Eng C*. 2016 Sept;66(1)130-137.
 43. K Huang FL, Yoshida Y, Nakabayashi H, Huang KP. Differential Distribution of Protein Kinase C Isozymes in the Various Regions of Brain. *J Biol Chem*. 1987 Nov;262(32):15714-15720.
 44. Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem J*. 1998 Jun;332 (Pt 2):281-292.
 45. Denning MF. Specifying protein kinase C functions in melanoma. *Pigment Cell Melanoma Res*. 2012 Jul;25(4):466-476.
 46. Mason SA, Cozzi SJ, Pierce CJ, Pavey SJ, Parsons PG, et al. The induction of senescence-like growth arrest by protein kinase C-activating diterpene esters in solid tumor cells. *Invest New Drugs*. 2010 Oct;28(5):575-586.