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In Vitro Evaluation of Poly (Lactic-co-Glycolic Acid)/Polyisoprene Fibres for Soft Tissue Engineering

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Abstract:

The polymeric blend of Poly (Lactic-co-Glycolic Acid) (PLGA) and Polyisoprene (PI) has recently been explored for application as stents for tracheal stenosis and spring for the treatment of craniosynostosis. From the positive results presented in other biomedical applications comes the possibility of investigating the application of this material as scaffold for tissue engineering (TE), acquiring a deeper knowledge about the polymeric blend by exploring a new processing technique while attending to the most fundamental demands of TE scaffolds. PLGA/PI was processed into randomly oriented microfibres through the dripping technique and submitted to physical-chemical and *in vitro* characterization. The production process of fibres did not show an effect over the polymer's chemical composition, despite the fact that PLGA and PI were observed to be immiscible. Mechanical assays reinforce the suitability of these scaffolds for soft tissue applications. Skeletal muscle cells demonstrated increases in metabolic activity and proliferation to the same levels of the control group. Human dermal fibroblasts didn't show the same behaviour, but presented cell growth with the same development profile as presented in the control group. It is plausible to believe that PLGA/PI fibrous three-dimensional scaffolds are suitable for applications in soft tissue engineering.

Keywords:

Cellprene, Fibroblast, Myoblast, PLGA, PI.

1. Introduction

The main goal of tissue engineering (TE) is to develop a new organ or tissue to facilitate restoration of biological functions.¹⁻³ While TE can be summarized to three approaches - cells and cellular substitutes; induction of tissue growth factors; cell seeding into a biomaterial scaffold - the third is the most common. However, several demands have been identified as crucial in scaffold manufacturing. These scaffold supports must present interconnected porosity, with pores in appropriate scale to facilitate tissue integration and vascularisation; must encourage cell adhesion, differentiation and proliferation through appropriate surface chemical properties; must be produced from materials that present controlled biodegradation/bioreabsorption, in a way that the new formed tissue will eventually substitute the scaffold. Furthermore, the material must be easily manufactured in a variety of shapes and sizes and present mechanical properties related to the implant site, altogether without inducing any adverse reactions.²⁻⁶

Fibrous scaffolds increase cell functionality if compared to "solid-wall" scaffolds, allowing the culture to generate a well organized tissue, closer to the structure presented by naturally aligned extracellular matrices, like muscle tissue. In order to obtain micro and nano fibres oriented in parallel or randomly, electrospinning has been widely investigated and applied. However, it shows limitations when it comes to obtain a defined porous structure uniformly interconnected required to allow tissue proliferation, as well as a micrometric space between fibres that facilitate cellular proliferation kinetics.⁷⁻¹³

With that in mind, the Dripping Technique¹⁴ was conceived as an alternative for micrometric polymeric fibres obtaining methods. It consists of the exposure of a dissolved polymer to an organic reagent, generating fibres by precipitation of polymers and simultaneous separation of its solvent under constant rotation. By controlling a polymer's exposition rate, precipitation reagent nature and speed of rotation, it is possible to control fibre diameter and morphological uniformity. Primarily used as mechanical reinforcement in calcium phosphate matrix applied for bone tissue engineering, these fibres were previously obtained in Poly (Lactic-co-Glycolic Acid) and Sodium Alginate, using methanol, ethanol and isopropanol as precipitation reagents, without alteration in the polymer's chemical groups during the process. For full separation of generated fibres from an alcoholic medium, lyophilisation was applied to said emulsion, providing dry fibres as a final result, which can be applied as reinforcement and possibly as scaffolds for soft tissue engineering.¹⁵⁻¹⁷

Poly (Lactic-co-Glycolic Acid) (PLGA) has been applied in the medical and pharmaceutical fields not only as a scaffold, but also as a suture thread, plates for bone fixation and stents. Due to its excellent in vivo properties, its behaviour profile became an excellent standard in those applications.¹⁸ However, since it is a mechanically fragile polymer, with low plastic deformation level, its application as an implant close to soft tissue may cause damage during treatment, specially the rip of tissues by its fragments generated during PLGA's bioreabsorption process.¹⁹ The blend between PLGA and Poly(isoprene) (PI) was summarily conceived from the need of manipulating these mechanical properties. Although the immiscibility between PLGA and PI has already been reported, and the original aim of this blend is to reduce PLGA fragility, it is plausible to hypothesize the in vivo bioreabsorption of PLGA, while PI remains in the body inducing a possible neovascularisation effect.²⁰⁻²¹ The blend of Poly (Lactic-co-Glycolic Acid) / Poly(Isoprene) was named CellpreneTM.^{22,23}

The first applications of Cellprene™ in vivo were focused on the treatment of tracheal stenosis. The application of polymeric stents includes prevention of lumen closure and obstruction of any tubular structure in the body i.e. blood vessels, urethra, trachea and bronchus.^{24,25} With that in mind, a spiral stent was manufactured from PLGA/PI blend, submitted to sterilization, and implanted in the trachea of New Zealand rabbits.²⁶

Cellprene™ was also evaluated in a bone environment, during studies aiming at more effective and less damaging treatment of craniosynostosis.²⁷⁻²⁹ Main treatment consists of osteotomy of these sutures and sequentially use of metallic springs for cranial expansion purposes.³⁰⁻³² The idea of replacing the metallic springs with polymeric springs seeks the application of a material that will be better tolerated and posteriorly absorbed by the body, avoiding the need of a second surgical procedure to extract the device.³³ Evaluating the cranial response in rats (Wistar) and rabbits (New Zealand), the springs made of PLGA/PI showed proper expansion of interbone space, biocompatibility with the bone environment and neovascularisation of areas surrounding the device.^{34,35}

From the positive results presented in other biomedical applications comes the possibility of applying Cellprene™ in tissue engineering, acquiring a deeper knowledge about this material by exploring a new processing technique while attending to the most fundamental demands of TE scaffolds. Its mechanical properties may suggest this material to be more reliable as a scaffold for reconstruction of soft tissues, such as muscle, cartilage and skin.²⁶ Therefore this study aims at obtaining the physical/chemical/biological characterization of fibrous scaffolds of a PLGA / PI blend.

2. Materials and Methods

2.1. Fabrication of Fibres and Scaffolds

Poly (Lactic-co-Glycolic Acid) (Purac Biomaterials – Holland) is a copolymer with proportions of 84 mol% monomer L-lactate and 16 mol% glycolide. Presenting pH of 7.2 and number-average molecular weight (M_n) of 250,000, PLGA was used as obtained, without previous purifications or processing. Poly(Isoprene) (Mafer – Brazil) presented $M_n=295,000$, and was neutralized with 2M HCl solution at an interval of pH 7.2-7.8 and submitted to a drying process. The polymer was purified by a re-precipitation method, using chloroform (CHCl_3) as a solvent, and re-precipitated in methyl alcohol (CH_3OH).³⁶ Chloroform (99.8%) (Synth – Brazil) and methyl alcohol (99.8%) (Synth – Brazil) were used as obtained, without previous purifications or processing. The materials were dissolved in chloroform in 60%PLGA/40%IR (w/w) proportion. Once homogenized, the

blend PLGA/PI was dried in an oven for 24 hours at 40°C to volatilize the solvent. Polymers were stored in a refrigerator at 4°C to avoid possible thermal degradation until processing period.

In order to obtain fibres, PLGA/PI blend was dissolved in Chloroform - concentration 1%w/v - and submitted to the dripping process. The blend was exposed to 500ml of ethanol (99.8%.) (Synth – Brazil), in mechanical rotation, exposure rate of 400ml/h, at room temperature. After precipitation, emulsion of fibres in ethanol were freeze-dried under vacuum environment in lyophilizer (Terroni Enterprise II - Brazil) for 24h at -40°C. Obtained fibres were then prepared in two different sample standards: three-dimensional scaffolds (3D) and two-dimensional slices (2D). For 3D samples, fibres (total weight: 1g) were shaped at room temperature into cylindrical scaffolds, measuring Ø12mm and height of 5mm. For 2D samples, obtained 3D scaffolds were sliced through their circular face into 5µm thick slices. Said slices were then mounted onto glass cover slips.

2.2 Polymer Thermochemical Properties

Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC) were applied to samples of pure PLGA, pure PI and PLGA/PI fibres. FTIR was performed using a Perkin Elmer Spectrum 1000 (USA) spectrometer, at room temperature, within the spectrum of region between 400-4000cm⁻¹. DSC was performed using a TA Instruments Q20 (USA) calorimeter, inert atmosphere, heating rate of 10°C/min, first heating cycle between 25°C and 190°C, and second heating cycle between -80°C and 190°C. Both FTIR spectrogram and DSC thermogram were generated using software Origin Pro 8 (USA).

2.3 Morphology of Fibres and Scaffolds

The density and porosity values of 3D scaffolds were measured by liquid displacement, based on the technique previously described by Zhang & Ma³⁷. A scaffold sample of weight W was immersed in a graduated cylinder containing a known volume (V1) of ethanol. The sample was kept in the ethanol for 5 min, and then a series of brief evacuation-repressurization cycles were conducted by a vacuum pump, to force the ethanol into the pores of the scaffold. Cycling was continued until no air bubbles could be observed emerging from the scaffold. The total volume of ethanol and the ethanol-impregnated scaffold then was recorded as V2. The volume difference, (V2 – V1) was the volume of the polymer skeleton of the scaffold. The ethanol-impregnated sample was

removed from the cylinder and the residual ethanol volume recorded as V_3 . The quantity $(V_1 - V_3)$ – the volume of the ethanol held in the pores – was determined as the void volume of the scaffold. Thus the total volume of the scaffold will be $V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3$. The density of the scaffold (d) will be expressed as $d = W/(V_2 - V_3)$. This evaluation was applied for 10 samples of 3D scaffold. In order to determine fiber diameter and inner space between fibers (pore size), each parameter was measured at 30 points randomly selected for 3D scaffolds. The fibre images were obtained in a Carl Zeiss Microscope (Germany), equipped with a Axio Cam ERc5s and the images processed with Axio Vision 4.8 software.

Mechanical properties of both scaffolds were determined using the Instron 3369 at room temperature, for compressive and tensile assays. A crosshead speed of 0,5 mm/min was applied for compression, while the speed of 0,1 mm/min was applied for tensile, both under a load cell of 2KN. This evaluation was applied for 10 samples of 3D scaffold. Stress-Strain curves were generated using software Origin Pro 8 (USA).

2.4 Cell Maintenance and Culture

In vitro cell culture over two-dimensional samples was produced in order to evaluate Cellprene's suitability to tissue engineering. Murine myoblasts (line C2C12) and Human Dermal Fibroblasts (HDF) were acquired from the European Collection of Cell Culture (Health Protection Agency - United Kingdom). Proliferative cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, U.K.) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 2mM L-Glutamine and 1% penicillin/streptomycin preparation (Invitrogen) at 37°C, 95% humidity and 5% CO₂. Cells were passaged when subconfluent - 80-90% - with Trypsin EDTA preparation (0.25%, Invitrogen), and medium was replaced every 2 days. This treatment was performed for both myoblasts and fibroblasts.

Immediately before cell culture, 2D samples and blank glass cover slips (TCP - control group) were placed into 12 well culture plates and sterilized by UV light direct exposure for 30 minutes in each side of the samples. All samples were seeded with 50,000 cells/well for a period of 30 minutes in 50µl cell culture medium to ensure maximum cell adhesion to fibres surface. The addition of 1.5ml medium to immerse the entire scaffold was performed. Medium was replaced every 2 days. All following *in vitro* assays were performed after 1, 3, 7 and 14 days of seeding.

2.5 Cell Metabolic Activity

Cell (C2C12 & HDF) seeded samples were examined for metabolic activity by alamar blue test. Initially for the determination of the standard graph, cell suspension was serially diluted and different densities of cells were plated on a tissue culture treated 24 well plate followed by a short incubation of 2 hours for the cells to attach to the surface. Media was gently aspirated from the wells followed by the washing of the well with sterile phosphate-buffered solution (PBS) three times. Alamar blue (89%PBS and 11% Resazurin (Sigma)) working solution (1 ml) was added to the wells followed by the incubation of the well plates for 2 hours at 37°C in a humid environment with 5% CO₂. From the whole reaction mixture, 200 µl was removed and transferred to the 96 well plate and plate was read at 600 nm. On the observation days, media from the seeded samples was aspirated followed by the washing of the scaffolds with cold PBS, addition of Alamar Blue working solution and incubation period of 2 hours at 37°C. After the incubation period, the solution was transferred to 96 well plate and colour developed was measured with a spectrophotometer at 510 nm (excitation) and 590 nm (emission) using the Fluostar Optima spectrofluorometer (BMG Labtech - Germany). In order to determine significance of results, data was treated using Analysis of Variance - ANOVA. In all instances, $p=0,025$ was considered as level of significance.

2.6 Cell Morphology

In order to observe cell (C2C12 & HDF) population and morphology, seeded samples were stained with Toluidine Blue (Sigma). In each observation period, cells were transferred to 12 well plates and gently rinsed with PBS to eliminate culture media traces. To fix cells 300µl of a PBS solution supplemented with 4% (v/v) p-formaldehyde was added to the wells. After 15 minutes of exposure, the fixing solution was removed and the samples rinsed with PBS. Each sample was then exposed to an aqueous solution of Toluidine Blue 1% (v/v) and finally rinsed with purified running water. Exposition time was 60 seconds for samples seeded with C2C12 and 30 seconds for samples seeded with HDF. The samples were observed and images obtained through a Leica DMRB microscope (USA) provided with QWin software.

2.7 DNA Assay

After washing with PBS, samples were transferred to a new plate and 200µl 1% (v/v) Triton X (Sigma) was added to the samples. After 10 minutes incubation at 37°C, the Triton X were deposited into Eppendorf tubes. These solutions were subjected to three

cycles of freezing at -80°C , defrosting at 37°C and centrifuge rotation, forming a precipitate mass in the Eppendorf, which was transferred to 96 well plate. The picogreen DNA assay kit (Invitrogen, UK) was applied to these precipitates according to the manufacturer's instructions. Serial dilutions of DNA standard (0 to 2 mg/ml) were used to construct a calibration curve. Briefly, 100 μl of sample or standard were incubated with 100 μl Quant-iT PicoGreen dsDNA reagent in the dark for 5 min at 37°C . After the incubation period, plates were measured with a spectrophotometer at 485 nm (excitation) and 520 nm (emission) using Fluostar Optima spectrofluorometer (BMG Labtech - Germany) for the estimation of ds-DNA accumulated over fibres. In order to determine significance of results, data was treated using Analysis of Variance - ANOVA. In all instances, $p=0,025$ was considered as level of significance.

3. Results and Discussion

3.1. Polymer Thermochemical Properties.

Analysis of the chemical groups present on PLGA, PI and PLGA/PI blend was possible through observation of FTIR spectra, as shown in Fig. 1a. This technique allowed verifying the possible occurrence of reactions between polymers and their interactions developed by the use of organic solvent during the blend obtaining process.³⁸

Knowing the molecular structure of the polymers, one might expect observation of groups C=O and C-O, related to PLGA, and bands related to CH_2 , C=C and =CH bonds, related to PI, as well as vibrations in the region of CH_3 bonds, a group relevant in both polymer chains. Symmetrical and asymmetrical stretching of CH_2 and CH_3 are observed between $2980\text{-}2850\text{cm}^{-1}$.^{39,40} These bands are noticed less intense on PLGA spectra when compared to PI spectra, presenting an intermediary intensity for PLGA/PI blend due to its composition consisting of 40% of Poly(Isoprene) and 60% for Poly(Lactic-co-Glycolic Acid).²⁶ Around 1663cm^{-1} , a discrete band is observed for PI, referring to the vibration of the C=C bond.⁴¹ The same band is almost imperceptible on the blend spectra, an event also observed due to reduced PI concentration in Cellprene. Stretching at 836cm^{-1} is also observed, corresponding to =C-H bending outside C-H bond plan.^{38,40} Bands correspondent to characteristic groups of PLGA appear in its spectra as well as in the blend's spectra in the same position and intensity. The elongation of C=O bond is recognizable by the acute and intense band in 1760cm^{-1} .³⁹ On the other hand, the characteristic stretching in bond C-O for aliphatic polyesters is indicated in 1185cm^{-1} and 1090cm^{-1} . While 1185cm^{-1} refers to saturated esters stretching C-C(=O)-O, 1090cm^{-1}

refers to O-C-C group.^{42,43} As expected, these bands are absent in PI spectra. By the observation of each spectra it is possible to assume that the use of organic solvent for blend obtaining didn't present an impact on the chemical bonds in the polymer.

In order to observe polymers thermal transitions, differential scanning calorimetry was performed. The melting temperature (T_m), a first order transition, with the formation of endothermic peaks, was observed during the first heating cycle. Glass transition temperature (T_g), a second order transformation, with dislocation of the thermogram base line, was observed during the second cycle.^{44,45} PLGA, PI and PLGA/PI blend thermograms are presented in Fig. 1b.

When in solid conformation, polymeric chains can present a random organization, formed by amorphous polymers, or a partially aligned organization, giving the polymer certain crystallinity. However there is never total crystallinity in these compounds, only regional. Therefore, polymers with crystalline regions are called semi-crystalline.⁴⁶ The transformations observed in thermograms are nothing but alteration in polymeric chains behaviour when exposed to thermal variations. With that in mind, the crystalline melting temperature of polymers is observed by a peak formation, as shown at 149.45°C on PLGA curve.⁴⁷ It is known as the temperature in which the system acquires enough energy to overcome secondary intermolecular forces between crystalline phase chains, affecting the consistent packing structure and conducting the material to a more viscous state.⁴⁸ This transition is characteristic of semi-crystalline polymers, and is absent in amorphous materials. There are no transitions of this order in the PI thermogram, which lends to belief that Poly(Isoprene) is an amorphous material. The thermogram of the blend at PLGA T_m region presents a subtle thermal variation. Once the peak's area corresponds to heat of fusion, a reduction in this area relates to a reduction in the necessary energy to overcome intermolecular forces in the material's crystalline region.⁴⁴ As the peak area is relevantly smaller in the thermogram of the blend, one may assume that the addition of PI into the blend would reduce the presence of crystalline regions and, consequently, the blend's crystallinity.

The glass transition temperature was obtained by applying the tangent rule.⁴² These second order transitions, related to the beginning of motion in the polymeric chains, were observed at -66°C for PI and 59°C for PLGA. The thermogram of the blend presented both PI and PLGA T_g at the same positions. By observing glass transition temperature in blends, it is possible to predict the miscibility between the polymers present in such blends. When a complete interaction happens, polymeric chains interpenetrate as if they were in the same structure, therefore generating a single T_g dislocation in the

thermogram.^{49,50} For partially miscible blends the thermogram present two variations in the curve's base line with visible approximation between glass transition temperatures. However, for immiscible blends, T_g is observed for each polymer in the same position observed in the raw polymer's thermogram.^{42,44} PLGA/PI blend, by presenting transitions at -66°C and 59°C may be considered immiscible in this blending proportion of materials.

3.2. Fibres and Scaffolds Morphological Analysis.

Assays were carried in order to obtain information around fundamental characteristics of fibres and scaffold prior to in vitro evaluation. Through microscopy it was possible to analyse the fibre diameters and the pore size, predicting the topographic conditions for cell growth into the scaffolds. Fibres obtained via the dripping technique presented a mean diameter of $20.85\ \mu\text{m} \pm 5.01$ and pore size of $75.47\ \mu\text{m} \pm 15.76$. Although it can be mathematically assured that the specific surface area of the network and the characteristic dimensions of interfibre voids are strongly influenced by the crosssectional morphologies of fibres⁵¹, different studies diverge in opinion when it comes to the influence of fibre diameter on cell behaviour. The use of nanofibres, as per example, allows prompt cellular adhesion but restrains the capacity of cell proliferation and tissue formation. One may even suggest that scaffolds containing micro and nanofibres are advantageous by the combination of the microfibre's mechanical properties and nanofibre's biomimetics.⁵² However, the increase in fibre diameter also provides an enhanced internal space between them due to spatial restriction.⁵³ This effect is well observed in fibres obtained by the dripping technique. Presenting a fibre diameter in micrometric scale, the scaffolds generate a considerable pore size, which may facilitate cell proliferation. It is important to mention that, when applying fibrous scaffolds in tissue engineering, fibre diameter must be a characteristic carefully correlated to the cell line that will be seeded.^{54,55}

As observed on microscopy of the fibrous structure in Fig. 2, the fibres presented a certain entanglement. This aspect is majorly due to the fact that with the dripping technique, fibres are obtained in a liquid turbulent environment, which complicate the collection of aligned fibres. This lack of parallel orientation between fibres may be discouraging for their application in tissue engineering of directionally organized tissues, like skeletal-muscle tissue.¹² Cells tend to respond to topographic parameters by altering orientation, mobility, growth and differentiation, activation and modulation of genetic expression.⁵⁶ However, by presenting a micrometric diameter and consequently higher

anchorage area, these fibres may also represent an increase in cell viability, proliferation and density in vitro, specifically in the first days of culture.⁵⁷

As expected, by presenting a micrometric fibre diameter and a considerable pore size, these PLGA/PI fibres also present a relatively large porosity: $75.53\% \pm 9.18$. This entangled displacement of fibres provides an interconnected porous structure, that favours the cellular freedom of migration into the scaffold when cultured. Once a large amount of inner space between fibres indicates a lower amount of polymer in the scaffold's total volume and in the density relation mass/volume, the relatively high porous structure also provides scaffolds with low density: $0.125 \text{ g/cm}^3 \pm 0.02$.

By incorporating salt in diluted PLLA, with posterior solvent volatilization and salt washing, Shi et al.⁵⁸ achieved scaffolds with 90% porosity. It was also reported that this parameter varied in direct relation to the amount of salt incorporated. In another situation, scaffolds of PLLA obtained by thermal induced phase separation (TIPS) presented 93% porosity.⁵⁹ However providing extremely high porosity, both scaffold obtaining techniques may limit porous interconnectivity by the generation of solid walls inside the scaffold. Fibres of PLGA with approximately 80% porosity were shown to be effective on regeneration of bone tissue.⁶⁰ Natural polymer based scaffolds, like electrospun collagen, presented relevant results in the regeneration of dermal fibroblasts, even presenting porosity inferior to 50%.⁶¹

With a good understanding of PLGA/PI fibrous scaffold morphology, its mechanical properties were also evaluated through axial compressive and tensile assay. The curves relating stress vs strain are presented in Fig. 3 for both compressive (Fig 3a) and tensile (Fig 3b) tests. This analysis allows evaluation of a possible behaviour presented by the scaffold in situ, and the modulus at yield for muscle and dermal tissues.⁶² Table 1 presents the results for the mechanical properties of the scaffolds.

In the compressive assay, the yield point of the curves was analysed, with exposition of its strain, stress and modulus. A low modulus at yield value, like the one presented by the scaffold, represents a lower capacity to tolerate stress without deform permanently. However, a high reading of stress at yield point indicates the scaffolds mechanical stability during its elastic transformation, a result that may be related to the entangled structure of fibres randomly organized. Different materials are constantly submitted to different processing techniques in order to generate ideal scaffolds for TE. PLGA scaffolds obtained by TIPS presented a compressive modulus at yield of approximately 3MPa and stress of 0.1 MPa at the same point. It was observed that the scaffold's modulus raise with the increase of polymer proportion in the solution previously

to processing, with respective increases in scaffold wall thickness.^{13,63} These modulus values are relatable to the results presented by PLGA/PI fibrous scaffolds. When a scaffold porosity was obtained through the insertion of gelatine microspheres into PLGA, also using TIPS as process, the scaffold modulus reduces to 0,4 MPa. Despite the low value, the interconnectivity and uniformity of porousness may help in minimizing structural defects and the eventual collapse of the scaffold.⁶⁴ The entangled structure of PLGA/PI fibres provides this structural stability, preventing the existence of micrometric flaws. Poly(Caprolactone) scaffolds have also shown results quite distinct when varying the obtaining method and, consequently, the scaffold's inner structure. Scaffolds obtained by additive manufacture presented a compression modulus of 1,8MPa, while fibrous scaffolds obtained through electrospinning presented an impressive modulus of 900MPa and stress at yield of 14MPa.^{65,66} Although the fibres had a randomly organized structure, the scaffold presented mechanical properties appropriate for applications under high mechanical demand, like bone tissue engineering. In the trabecular bone, a region with high porosity, modulus value reaches around 500MPa and compressive stress of 7-10MPa.⁶⁵ As the mechanical properties represent a major role in bone tissue engineering, PLGA/PI scaffolds are not appropriate for this application.

When evaluated the PLGA/PI fibrous scaffold response to tensile strength, higher values of strain were noticed. At maximum stress, the high values of deformation may indicate the scaffolds capability to resist plastic deformation until its tensile strength resistance limit. The results are also relatable to the entangled structure of fibres on the scaffold.⁷ By observing the strain vs. stress curve profile, it is possible to realize that the scaffold reaches its limit of stress and keeps the level of force per area until its collapse. This reinforces the idea of a well structured scaffold that doesn't collapse gradually during tensile application. For soft tissues, mechanical strength does not present itself as a vital propriety. The replacement of, for example, muscles, cartilage and skin take into consideration elasticity, once the scaffold elastic structure is a strong determinant for resilience, texture and tissue generation. Scaffolds obtained by the combination of synthetic human elastin and collagen reached Young's modulus values lower than 1MPa and were considered appropriate for dermal tissue engineering.⁶¹ On the other hand, human femoral artery, well known for being one of the biggest arteries of the human body, when submitted to the mechanical evaluation it presented modulus around 9MPa and maximum stress at 1MPa.⁶⁷ Skeletal muscle, a soft tissue when contracted, presents its modulus between 12-15kPa (0.012-0.015MPa), while fibroblasts present an even lower

modulus, between 2-5kPa (0.002-0.005MPa).⁶² These values may suggest the PLGA/PI scaffold suitability for application in soft tissue engineering.

3.2. *In vitro* evaluation.

Two-dimensional scaffolds were seeded with either murine myoblasts C2C12 or human dermal fibroblasts. For both cell lines, the observation period was 1, 3, 7 and 14 days, when analysis of metabolic activity, morphology and proliferation (DNA quantification) were conducted. Fig. 4 shows (a) metabolic activity and (b) double-strained DNA quantification for myoblasts culture. Optical microscopy of such cell culture is shown in Fig.5.

For C2C12 cell line, the counts were found to be slightly higher in the control group for population, metabolic activity and dsDNA, in all periods of observation. However, there is no significant difference in proliferation and DNA between groups after 14 days of culture. Both treated and control groups present a similar growth profile, being the initial number of cells seeded restored after three days. Cellular population increases to six times the initial population counting when the interval between one and 14 days was clearly observed in the optical microscopies.

Skeletal-muscle tissue engineering has been extensively studied as an alternative for autograft, allograft and xenograft applications. However, it has been pointed out that scaffolds for muscle tissue growth need a parallel aligned structure in order to induce the development of immature muscle fibres, also known as myotubes. Additionally, the redevelopment of muscle tissue also depends on mechanical and electrical stimuli *in vitro*, cell culture exposition to specific growth factors, effective vascularisation and subsequent development of its neural structure *in vivo*.⁶⁸ The fusion of myoblasts into myotubes shows to be effective in aligned micro and nanofibres due to this topographic and spatial configuration, thus the cells capability to move is directly related to morphology, orientation and extracellular matrix production.⁶⁹

But culture of myoblasts over unorganized topographies can also demonstrate a certain material potential of application in muscle tissue engineering. Aviss et al. (2010) compared the growth levels of myoblasts into PLGA fibres obtained through rotational collection electrospinning with different speeds of rotation. It was observed a distinct cellular morphology by varying the fibrous topography. Fibres aligned in parallel induced myoblasts in a more elongated shape, while randomly organized fibres induced the cells to a polygonal shape, the same configuration presented by cells in the control group (glass coverslips). Even so, cell counts presented similar results for both fibres, which leads to

believe that, for PLGA/PI fibres, an alteration in the fibrous morphology could result in a new morphologic configuration for the myoblasts without compromising its proliferation capacity.⁷ Agarose-gelatin-chitosan scaffolds, even without an organized structure for myotubes differentiation, were found to be effective in muscle tissue engineering. Observed cell growth in the same levels of control group indicate the material as adequate for cardiac muscle development, once the appropriate porosity guarantee an effective cellular mobility and consequent production of ECM.⁷⁰ These conditions, similar to those observed on PLGA/PI fibres, may represent the material's adequacy to cardiac tissue regeneration. The development of new muscles can also be achieved through myoblast and fibroblast co-culture, allowing for the proliferation of more complex systems through the viability of both cell lines in the same material.^{68,71}

Fig. 6 shows (a) metabolic activity and (b) double-strained DNA quantification for fibroblasts culture. Optical microscopy of such cell culture is shown on Fig.7. As it was observed for the C2C12 cell line, fibroblast readings for proliferation and dsDNA were shown to be higher in the control group, with the initial population re-established after three days of culture. On the other hand, growth profiles are not directly comparable. Despite a significant growth observed for fibroblast over PLGA/PI fibres after seven days, in the same period the difference of proliferation between treated and control groups is already significant, behaviour extended until 14 days of culture. The last point of observation compared to day one points out a significant cell growth for both groups, however after 14 days the cellular population in the control group is about seven times higher than the observed for culture over fibres. Such expressive difference in population is clearly observed in the microscopic images, where it is possible to realize a high confluence of fibroblasts over control group after seven days, behaviour not replicated in the treated group. When it comes to differentiation, the elongated shape of fibroblasts is observed in the control group after three days and in the treated group after 14 days, which leads to belief that the culture over fibres potentially delays fibroblast growth and proliferation, although does not prevent said differentiation and consequent proliferation.

Studies conducted with PLGA electrospun fibres evaluated this material's viability as a substitute of ligaments through tissue engineering. The culture of fibroblasts was submitted to various topographic environments, with fibres either parallel or randomly oriented. It was observed that fibroblasts did not present sensibility to fibre orientation and most effective cell growth was related to the fibre diameter, especially due to the fibre's ability to facilitate anchorage and allow cell proliferation.⁵⁷ One may consider the fibroblasts development to a more elongated format characterizes an effective cell

differentiation, as observed for the culture over PLGA/PI fibres after 14 days. Even in situations where the cell growth doesn't achieve the same levels as the control group, the scaffolds are still conducted to more extensive studies. This happens when fibroblast proliferation in culture presents a meaningful growth profile after two weeks, as well as an elongated morphology in the cells observed individually.⁷²⁻⁷⁴ Said behaviour is observed in the fibroblasts cultured over PLGA/PI fibres, reinforcing the viability of these fibres for tissue engineering, even presenting cell population growth in a slower pace compared to control group.

Being naturally found in connective tissues, fibroblasts are explored in several applications in TE, from skin and muscles to tendons and ligaments.^{57,61,75} Skin reconstitution through tissue engineering, for example, depends on the presence self-renewal keratinocytes and a functional dermal substitute that have proper cellular and acellular components. In other words, an effective scaffold that minimizes scarification. For that to be achieved, the use of angiogenic factors is essential in order to guarantee fast vascularisation on the treated area.⁷⁶ As previously stated, *in vivo* applications of PLGA/PI blend have been related to the formation of intensively vascularised areas in bone tissue reconstruction.^{34,35} This effect is strongly related to the presence of Poly(Isoprene) in the grafts, as previously observed in other applications.⁷⁷⁻⁷⁹ This may indicate another desirable characteristic of this polymeric blend for dermal tissue engineering.

Conclusions

The polymeric blend of Poly (Lactic-co-Glycolic Acid)/Poly(Isoprene) - Cellprene - was processed into microfibres through the dripping technique and submitted to evaluation around its application as scaffolds for tissue engineering. The production process of fibres did not show an effect over the polymer's chemical composition, despite the fact that PLGA and PI were observed to be immiscible. The structure of obtained fibres presented high porosity, large diameter of fibres and convenient pore size and distribution. These fibres in this morphology also supported an increase in cell viability, proliferation and density in vitro due the tangled structure and interconnected pores. Mechanical assays reinforce the suitability of these scaffolds for soft tissue applications. Skeletal muscle cells were cultured on a two-dimensional structure of such fibres and the cell line C2C12 demonstrated increases in metabolic activity and proliferation to the same levels of the control group, aside from clear demonstration of extracellular matrix formation. Human dermal fibroblasts didn't show such high readings, but presented cell growth with the same

development profile as presented in the control group. Further research is required in vitro, but it is plausible to believe that these PLGA/PI fibrous three-dimensional scaffolds are suitable for applications in soft tissue engineering.

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Figure 1: (a) FTIR spectra and (b) DSC thermogram of PLGA, PI and PLGA/PI blend.

Figure 2: Optical microscopy of PLGA/PI scaffolds fibrous structure.

Figure 3: stress (σ) vs. strain (ϵ) curves for (a) compressive and (b) tensile assays.

Figure 4: Myoblasts C2C12 (a) metabolic activity and (b) ds-DNA assay. Dashed line represents initial population. *represents statistically significant difference between indicated groups - ANOVA ($p=0,025$).

Figure 5: Optical Microscopy of myoblasts cultured on PLGA/PI fibres and control group (TCP), stained with Toluidine Blue. Scale Bar (10x): 200 μm . Scale bar (40x): 50 μm .

Figure 6: Human Dermal Fibroblasts (a) metabolic activity and (b) ds-DNA assay. Dashed line represents initial population. *represents statistically significant difference between indicated groups - ANOVA ($p=0,025$).

Figure 7: Optical Microscopy of human dermal fibroblasts cultured on PLGA/PI fibres and control group (TCP), stained with Toluidine Blue. Scale Bar (10x): 200 μm . Scale bar (40x): 50 μm .

Table 1: Mechanical properties of PLGA/PI fibrous scaffolds and soft tissues.

ASSAY	PROPERTY	SCAFFOLD	SKELETAL MUSCLE (C2C12) ⁶²	HUMAN DERMAL FIBROBLAST ⁶²
COMP.	Stress at Yield (MPa)	0.057		
	Strain at Yield (%)	1.5190		
	Modulus at Yield (MPa)	3.790		
TENSILE	Maximum Stress (MPa)	0.03063		
	Strain at Maximum Stress (%)	3.4590		
	Stress at Break (MPa)	0.03046		
	Strain at Break (%)	6.3070		
	Modulus at Yield (MPa)	2,715475	0.012-0.015	0.002-0.005

This represents the author accepted manuscript.