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Poly Lactic Acid Based Scaffolds for Vascular Tissue Engineering

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A critical obstacle encountered by tissue engineering is the inability to maintain large masses of living cells upon transfer from the in vitro culture conditions to host in vivo. Capillaries, and the vascular system, are required to supply essential nutrients, including oxygen, remove waste products and provide a biochemical communication “highway”. The successful use of tissue-engineered constructs is currently limited to thin or avascular tissues, such as skin or cartilage, for which post-implantation neo-vascularisation from the host is sufficient to meet the demand for oxygen and nutrients. To succeed in the application of tissue engineering for bigger tissues, such as bone or muscle, the problem of vascularisation has to be solved. Another task in this research field is the possibility to tune the biodegradability of the scaffold. After implantation, the scaffold must be gradually populated by cells and replaced by extra cellular matrix; with this respect, it is crucial that this replacement takes place with appropriate dynamics and a well-defined timescale. A premature degradation, in fact, could lead to a collapse of the structure as the newly generated tissue could not have reached yet the suitable mechanical properties. Conversely, a long degradation time could delay or completely interrupt the development of the new tissue. In this work scaffolds for vascular tissue engineering (VTE) were produced and characterized, utilizing several PLLA/PLA blends (100/0, 90/10, 75/25 wt/wt) in order to tune the biodegradability of the scaffolds. Cell culture into the scaffold were carried out and the non-cytotoxicity of "scaffolds", adhesion and cell proliferation inside them were evaluated. The results have shown that the scaffold do not induce cell toxicity; cells are able to grow into the tubular shape scaffold covering its internal surface, so they can be considered suitable for the application for the designed aimed.

1. Introduction

Cardiovascular disease remains the leading cause of mortality in western nations, with an estimated prevalence of almost 80 million in the USA alone . In particular, coronary artery disease is the leading cause of death, accounting for53%of the total mortality related to cardiovascular disease (Soletti et al., 2010).
Despite a clear clinical need for a functional arterial graft, success has been limited to arterial replacements of large-calibre vessels such as, the thoracic and abdominal aorta, arch vessels, iliac, and common femoral arteries. Currently, occluded vessels with diameters below 6mm are bypassed with autologous native blood vessels, such as the saphenous vein. However, those surgical

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techniques, which account for a majority of the demand, have generally proved inadequate, largely because of acute thrombogenicity of the graft, anastomotic intimal hyperplasia, aneurysm formation, infection, and progression of atherosclerotic disease (Conte, 1998) Regenerative medicine and, in particular, tissue engineering approaches are being investigated as potential solutions to these problems. To this end, autologous vascular cells are seeded in biodegradable (tubular) scaffolds which are subsequently cultured in vitro or immediately implanted. The ideal scaffold should be biocompatible, flexible, elastic, and biodegradable. To facilitate formation of vascular tissue, the porous scaffolds should provide a three-dimensional space for adhesion and proliferation of cells, allow diffusion of nutrients and metabolic waste products, and maintain suitable mechanical properties until maturation of the newly formed tissue(Song et al., 2010).

The fundamental difference between traditional vascular grafts and tissue engineered vascular grafts (TEVGs) based on scaffolds is biodegradation of the scaffold material used. TEVGs thus depend on the capacity of donor- and host-derived cells to secrete and remodel the scaffold, concurrently replacing it with extracellular matrix (ECM), forming a native-like structure.

A further crucial aspect of the tissue engineering regards the scaffold biodegradability. Biodegradable materials, like polymers, can decompose naturally, but their degradation products will remain inside the human body. As for bioresorbable materials, they will degrade after a certain period of time after implantation, and non-toxic products will be produced and eliminated via metabolic pathways. For the chemical degradation, two different modes are defined, they are (i) hydrolytic degradation or hydrolysis, which is mediated simply by water and (ii) enzymatic degradation which is mainly mediated by biological agents such as enzymes. Scaffolds should be biodegradable allowing extracellular matrix (ECM) to occupy the void space when the biomaterial is degraded. The rate of degradation, however is determined by factors such as configurational structure, copolymer ratio, crystallinity, molecular weight, morphology, stresses, amount of residual monomer, porosity and site of implantation (Cheung et al., 2007). The success of aliphatic polyesters in tissue engineering relies largely on their degradability and biocompatibility, as well as their good processability and mechanical properties.

PLA is a biodegradable thermoplastic polyester that can be produced through ring-opening polymerization of lactic acid. Since lactic acid is a chiral molecule, it exists in two forms, D-PLA and L-PLA. It is known that the properties of PLA are highly affected by the stereo-isomeric L/D ratio of the lactate units. In fact, PLLA and poly(d-lactide) (PDLA) consisting only of L- and D-lactate units, respectively, are highly crystalline with identical chemical and physical properties, while poly(DL-lactide) (PDLLA) consisting of racemic lactate units is completely amorphous. The involvement of D- and L-units in the sequences of PLLA and PDLA, respectively, gives a profound effect on their thermal and mechanical properties. Generally, the increased stereo-isomeric ratio decreases the cristallinity. For the aforementioned reason the polymer blend of PLLA and PDLLA is also an effective method for controlling the polymer crystallization, morphology, and hydrolysis nature (Tsuji and Ikada, 1996).

The goal of this study was to produce PLLA/PLA blends based tubular scaffolds in order to tune the initial level of the crystallinity, thus controlling the biodegradation rate. Moreover in order to verify the suitability of these products for tissue engineering application cell culture tests were performed in order to evaluate the non-cytotoxicity of scaffolds and the cell adhesion and proliferation

2. Experimental

2.1 Materials

Poly-L-lactic-acid (PLLA, ResomerTN) and Poly lactic acid were kindly supplied by Boehringer Ingelheim Pharma KG. The molecular weight of the polymers was calculated by measuring their intrinsic viscosity. The intrinsic viscosity was evaluated with a Ubbelohde capillary viscometer.

The Mark-Houwink constants to calculate the Mw were obtained from literature (Garlotta, 2001). The molecular weights of PLLA and PLA were respectively 114000 and 175000 Kg/Kmol.

2.2 Scaffold preparation

The vessel-like scaffolds were obtained by performing a Diffusion Induced Phase Separation (DIPS) process, after a dip coating, around a nylon fibre with a diameter of ~700 μm. Briefly, the fibre was first

immersed into a PLA/dioxane or PLA/PLLA blend/dioxane solution (dip coating bath) at a constant temperature (35 °C). Then the fibre was slowly pulled-out at different constant rates (from 10 to 40 cm/min) from the solution and immersed into a second bath (DIPS bath), containing pure water at the same temperature, for 10 minutes. Finally, the fibre was extracted from the bath, eventually rinsed in distilled water and dried at 80 °C for 48 h.
The scaffolds as obtained were analysed by scanning electron microscopy (SEM) with a Philips 505 Microscope on sample cross section fractured in liquid nitrogen and gold stained.

2.3 Cell culture and observation

The ECV304 continuous human endothelial cell, from ECACC (European Collection of Cell Culture), was cultured in Medium 199 (EuroClone) supplemented with 2 mM L-glutamine, 1 unit/ml penicillin, 10 mg/ml streptomycin and 10% Fetal calf serum (FCS) (EuroClone).
ECV304 cell solubilized in an extracellular matrix were seeded inside the PLLA scaffold 2,5 cm long, at 15 X 10^4 cell/cm. Media were exchanged every 2/3 days. Respectively after 14 days scaffolds were extracted from culture medium, fixed in a solution of 4% phormaldeyde for 10 min at room temperature and washed three times in PBS containing Ca^{++} and Mg^{++}. Tubes were sectioned across the transverse direction in smaller pieces about 8-10 mm long, longitudinally opened. To evaluate the presence of apoptotic cells and, therefore, possible effects of cytotoxicity by the scaffolds, cells were stained with a solution of Acridine Orange- Ethidium Bromide [100 µg/ml] in PBS for 10". Evaluations were performed by confocal microscopy observation (Olympus 1X70 with Melles Griot laser system, having two independent lasers).
For the Scanning Electron Microscopy (SEM) observation, the samples were fixed following the same procedure as described above, rinsed in PBS 1X and dehydrated in increasing concentrations of ethanol. Dehydrated samples were gold sputter coated and observed by using a PHILIPS 505 SEM.

3. Results and discussion

3.1 Morphology of the scaffolds

Some preliminary attempts to prepare scaffolds at various temperatures (60, 45 and 35 °C) were carried out at different fibre extraction rates (from 10 to 30 cm/min). Unfortunately, no homogeneous tubular scaffold were successfully produced at 45 and 60 °C at all the fiber extraction rates tested and polymer concentrations tested.
The poor and imperfect adhesion of the Polymer/dioxane solution to the nylon fiber at those temperatures could be probably related to the lower viscosity of the solution at high temperatures. On the other hand, when lowering the process temperature to 35 °C, homogeneous scaffolds were obtained.
Figure 1 reports cross sections of the scaffolds obtained by utilizing an 8% polymer/dioxane solution at different fiber extraction rates. As shown by the SEM micrographs, in both cases a significant porosity was detected in the wall of the tubular samples . However it was not possible to extract the nylon fibre out of the samples because of their fragility due to their low thickness (about 20 microns).
Figure 2 shows the cross sections of some samples prepared by utilizing a 10% polymer/dioxane solution. Also in this case an open-pore structure was observed along the wall of the scaffolds; by looking through the micrographs it is easy to notice a substantial increase of the thickness of the scaffold with respect to those described above (100 -200 microns). Moreover thickness increases when raising the fiber extraction rate. This result is in agreement with a previous work (La Carrubba et al., 2010), where it was showed that one of the more important parameters that strongly influences the final wall thickness of the scaffolds is the fibre extraction rate.

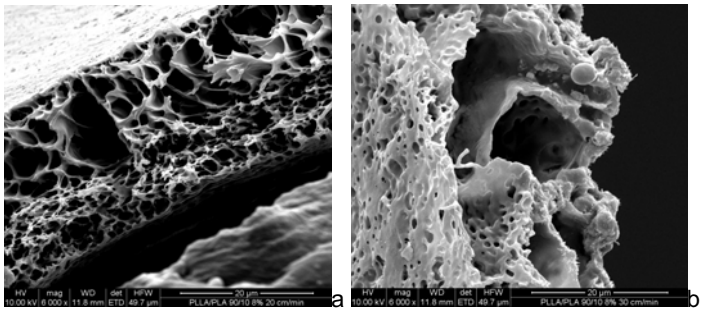

Figure 1: SEM micrographs of cross sections of PLLA/PLA 90/10 tubular scaffolds prepared from a 8% wt polymer/dioxane solution at different extraction rates; 20 cm/min (a); 30 cm/min (b).

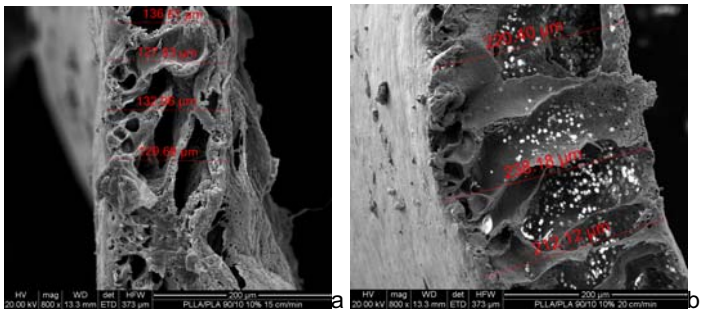

Figure 2: SEM micrographs of cross sections of PLLA/PLA 90/10 tubular scaffolds prepared from a 10% wt polymer/dioxane solution at different extraction rates; 15 cm/min (a); 20 cm/min (b).

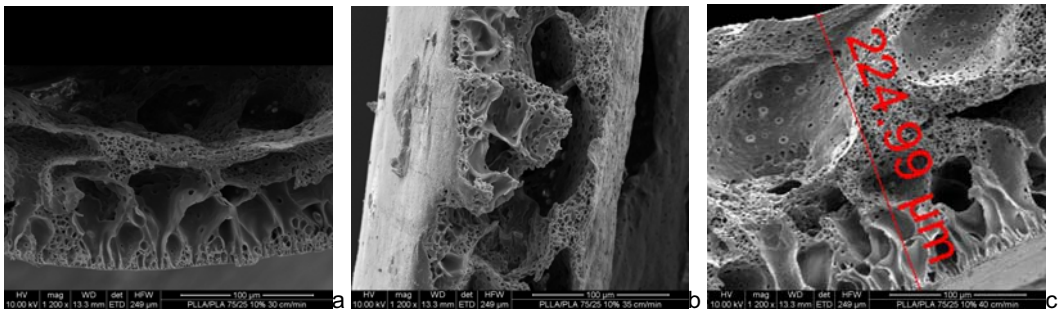

Figure 3: SEM micrographs of cross sections of PLLA/PLA 75/25 tubular scaffolds prepared from a 10% wt polymer/dioxane solution at different extraction rates; 30 cm/min (a); 35 cm/min (b); 40 cm/min(c).

Figure 3 reports cross section of scaffolds prepared starting from a 10% polymer/dioxane solution, increasing the PLA content in the Blend (from 10 to 25% wt) at different extraction rates (from 30 to 40 cm/min). Also in this case an open-pore structure was detected. The thickness of the porous walls for 90/10 75/25 PLA/PLLA scaffolds are shown in figure 4, from which it is possible to notice in both cases an increasing of the thickness by raising the fiber extraction rate.
However, in order to obtain easy-to-handle samples (thickness > 100 microns) when utilizing a 75/25 PLLA/PLA solution, the fiber extraction rate has to be increased with respect to 90/10 PLLA/PLA samples.
This could be reasonably explainable by recalling that a viscosity of the polymer/dioxane solution significantly decreases when passing from 10 to 25% in PLA content.

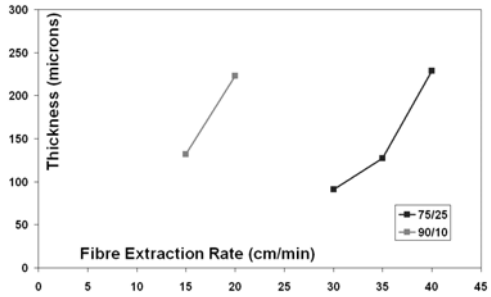

Figure 4: thickness of the tubular scaffolds in function of fibre extraction rate.

3.2 ECV culture

A cell culture inside the vessel-like scaffolds was carried out, by using ECV304 endothelial cells, which are the solely components of capillary bed and the first to form during embryonic development. Figure 5 illustrates a laser confocal microscope image of a longitudinal section of a scaffold with ECV304 cell grown for 14 days stained with Acridin Orange. As is possible to observe, cells show a good level of adhesion to the polymeric substrate. Moreover, the typical apoptotic cell morphology was not detected (figure 5 b). These results confirms the biocompatibility of our devices.

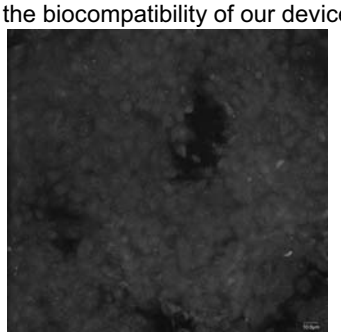

Figure 5: confocal microscope image of the ECV grown inside the scaffold for 14 days (b)

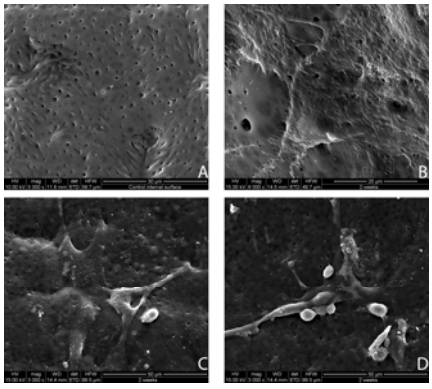

Figure 6: SEM micrographs of internal surface of the scaffold without cell (a) and after 14 days of culture (b, c and d).

SEM analysis has shown that the cultured cells were able to keep contacts each others, thus forming an homogeneous monolayer on the internal surface of the scaffolds.

4. Conclusions

Tubular scaffolds for tissue engineering applications based on PLLA/PLA blends were produced. Two different polymer ratios were utilized in order to tune the crystallinity of the scaffolds and the biodegradation rate. The as-produced scaffolds present an open structure with interconnected pores along the wall. Moreover, it was possible to change the thickness of the wall by varying a simple experimental parameter (the rate at which the fibre is extracted from polymer/dioxane bath). Preliminary biological cultures in the scaffolds with Endothelial Cells have shown a good level of adhesion and proliferation with a development of an homogeneous “vessel-like” monolayer and have confirmed the non cytotoxicity of the materials utilized. These results indicate these products as promising devices to be employed in vascular tissue engineering field.

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