**Bio-safe processing of polylactic-co-caprolactone and polylactic acid blends to fabricate
nanofibrous porous scaffolds for tissue engineering**

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Abstract

In this study, the design and fabrication of porous scaffolds, made of blends of polylactic-co-caprolactone (PLC) and polylactic acid (PLA) polymers, for tissue engineering applications is reported. The scaffolds are prepared by means of a bio-safe thermally induced phase separation approach with or without the addition of NaCl particles used as particulate porogen. The scaffolds are characterized to assess their crystalline structure, morphology and mechanical properties, and the texture of the pores and the pore size distribution. Moreover, *in vitro* human mesenchymal stem cells (hMSCs) culture tests have been carried out to demonstrate the biocompatibility of the scaffolds. The results of this study demonstrate that the nano and macro-structural properties of the scaffolds strongly depend on the polymer crystallization, which, in turn, is affected by the blend composition. Indeed, neat PLC and neat PLA crystallize into globular and randomly arranged nano-size scale fibrous conformations, respectively, thus addressing their pore structure features and mechanical response. Concomitantly, the addition of NaCl particles during the fabrication route allows for the creation of an interconnected network of large pores inside the primary structure. Finally, the results of cell culture tests demonstrate that both the nano and macro-structure of the scaffold affect the *in vitro* hMSCs adhesion and proliferation.

Keywords: Crystallization; Nanofibrous scaffold; Phase separation; Polymer blend; Stem cells.

1 Introduction

The successful regeneration of biological tissues and organs is an important challenge for the tissue engineering (TE) community. To date, the design and fabrication of biocompatible and biodegradable porous scaffolds represents the most useful TE approach to attain this objective. Indeed porous scaffolds serve as a synthetic analogue of the extracellular matrix (ECM) of native tissues by providing the biochemical and biophysical stimuli essential for appropriate cell growth and new tissue development [1-3].

The rational design of porous scaffolds mimicking the structure and functionalities of ECM relies on the fact that, natively, the cross-talk between cells and ECM determines the correct tissue development and functionality [4]. From a morphological and structural point of view, biomimetic scaffolds must be designed having high porosity and interconnected pores of several hundreds of microns in size, to enable cell colonization and de-novo ECM biosynthesis [5,6]. However, in biological tissues, the ECM is also composed of an intricate interweaving of protein fibres, mainly collagen and elastin, ranging from 10 to several hundreds of nanometres. These nanofibers are covered with nanoscale adhesive proteins, such as laminin and fibronectin, which provide the biochemical and biophysical guidance for cell adhesion, growth, migration and differentiation [7].

Macroporous materials with a nano-size fibrous microscale have been reported to be optimal scaffolds for *in vitro* cell culture and *in vivo* new tissue regeneration [7-12]. This is because nano-size scale fibers provide a biomimetic structure replicating the morphological and textural features of native ECM [7], improve the adsorption of proteins necessities for cell adhesion [10], act as a physical guidance for cell migration, growth and morphogenesis [8,9] and stimulate cell differentiation and implant vascularization [11,13]. The relevance of the nano-size scale fibrous architecture has been widely demonstrated in the case of polycaprolactone (PCL) scaffolds in terms of adhesion/proliferation [8] and cell metabolism [9] and, in the case of poly(lactic acid) (PLA) scaffolds for their ability to promote bone [10,11] or nerve regeneration [12]. Porous scaffolds

made of PLC copolymers of different compositions have been used for blood vessels [14,15], bone [16], cartilage [17,18] and nerve regeneration [19] among others. This is because PLC chemical structure combines the high elasticity and low glass transition temperature of PCL with the stiffness of PLA at body temperature into a single biocompatible and biodegradable material [15].

Ma and co-workers [10,11] developed a combined approach, based on thermally induced phase separation (TIPS) and porogen leaching (PL) techniques, for controlling the nano-scale pore wall fibrous texture and macro-structure of PLA scaffolds, which enhanced protein adsorption [10] and *in vivo* new bone formation [11]. Phase separation from a polymer–solvent solution is based on the thermodynamic de-mixing of the system into polymer-rich and polymer-poor phases, which can be caused by antisolvent addition, using either conventional liquids or supercritical fluids [20,21], or by cooling down the solution below a binodal solubility curve [22,23]. This last approach corresponds to the TIPS approach and allows for the large-scale formation of nanometre-scale fibrous structures with characteristic diameters in the 50–500 nm interval, controlled by selecting the appropriate polymer/solvent combination, the cooling temperature and the process kinetics [24]. TIPS leads to a fibrous microstructure, i.e., nanofibres are arranged forming the pore walls (nano-scale fibrous structure), while the macrostructure does not have a fibrous appearance [25]. Beside TIPS, electrospinning and molecular self assembly are two alternative processes that have been developed for fabricating scaffolds composed of interwoven nano-scale fibres. Electrospinning uses an electric field to control the deposition of fibres on a target substrate starting from a polymeric solution or polymer melt [26,27]. This technique can be applied to a wide range of polymers and has the advantage of the possibility of achieving a high precision in the deposition of the fibres onto the collector's surface. Recently, attention has been devoted to the improvement of the porosity of fibre mats, as well as the control of the three dimensional distribution of the fibres for enhancing cell colonization and response [28,29].

Molecular self-assembly takes advantage of the organization of amphiphile peptides, a chemical compound possessing both hydrophobic and hydrophilic properties, into nano-size scale fibrous scaffolds possessing a fibre diameter as small as 10 nm [7,30]. These scaffolds are able to provide molecular functional motif sequences that promote adherence, differentiation and maturation of cells. Conversely, the hydrogel structure of the peptide amphiphile scaffolds limits the *in vivo* implantation into load bearing sites.

Biodegradable polyesters, such as PLA, PCL and their copolymers polylactic-co-caprolactone (PLC) are widely used for the fabrication of TE scaffolds with desired morphology and tailored mechanical functionality [3,5,12,31-33]. Furthermore, scaffolds prepared starting from these synthetic materials have good *in vitro* biocompatibility [5,31,32] and may degrade into non-toxic components with a controllable degradation rate during *in vivo* implantation [33].

The possibility to use a novel bio-safe TIPS process for preparing nanostructured PCL particles [34] and porous PLA scaffolds [32] by using ethyl lactate (EL) as green biocompatible solvent has been recently explored in our lab. As a further development, the aims of this work are (i) to blend PLA and PLC polymers to control the nano-size scale fibrous structures of porous scaffolds prepared by means of the TIPS; (ii) to combine TIPS and NaCl leaching techniques for achieving a pre-defined network of interconnected large pores; and (iii) to characterize the morphological, structural and *in vitro* biocompatibility properties of designed scaffolds.

2 Materials and methods

2.1 Materials

PLA (polylactic acid 80/20 L/DL ratio, molecular weight equals to 200 kDa and inherent viscosity of 3.8 dL/g) and PLC (polylactic-co-caprolactone 70/30 L-lactide/caprolactone co-polymer, molecular weight equals to 130 kDa and inherent viscosity of 1.57 dL/g) were provided by Purac Biochem (Gorinchem, The Netherlands). EL (ethyl lactate photoresist grade; purity $\geq 99.0\%$) and ethanol (99.5%) were provided by Sigma-Aldrich (Madrid, Spain). NaCl (sodium chloride) particles (Carlo Erba, Milan, Italy) were used as the leachable porogen.

2.2 Porous scaffolds fabrication by means of TIPS and TIPS combined with NaCl leaching

Porous scaffolds made of PLC, PLA and their blends were fabricated by means of TIPS and TIPS combined with NaCl leaching. In the TIPS process, the polymers were dissolved in EL at an overall concentration of 10 % (weight of polymer with respect to the solvent volume), by means of magnetic stirring at 70 °C for 6 h. The polymeric solutions were subsequently dropped in cylindrical aluminum moulds (d=5 mm, h=15 mm) and cooled down to -15 °C for 3 h to induce TIPS and solution gelation. The gels were soaked in excess of cold ethanol (-15°C) overnight to extract the EL and to induce the setting of the scaffolds avoiding the collapse of the polymeric structure. The samples were then washed with cold water and this step was repeated three times before freeze-drying processing. As shown in Table 1, four different compositions were selected, namely neat PLC, neat PLA and two PLC/PLA blends, PLC-90 and PLC-50 containing 90 and 50 % in weight of PLC, respectively.

In a different set of experiments, the TIPS process was combined with NaCl particles leaching. In particular, 2.5 g of polymeric solution were manually mixed with 4 g of NaCl particles, previously heated to 100 °C. The mixture was placed in cylindrical aluminum moulds (d=5 mm, h=15 mm) and kept for 30 s at room temperature, without any moving, to promote the sedimentation of the NaCl particles and their contact inside the polymeric solution. The mixture

was then processed by means of TIPS as previously described, although in this case the samples were further soaked in water for 1 week before freeze-drying in order to remove NaCl particles.

2.3 Morphological and structural characterizations

The crystalline structure and melting behavior of the scaffolds were characterized by means of X-ray diffraction and differential scanning calorimetric (DSC) tests. For X-ray analysis, the samples were tested on a XRD Rigaku Rotaflex RU-200 B, by using the Cu K_{α1} radiation in the 10–50° 2θ range and a scan step of 0.05°. For calorimetric analysis the scaffolds were tested on a DSC8500 apparatus (Perkin Elmer, Massachusetts, USA) equipped with a liquid N₂ controller CRYOFILL. Samples were first equilibrated at 10 °C for 5 min and then, heated up to 150 °C at a scanning rate of 10 °C min⁻¹ under an inert atmosphere.

Scanning electron microscopy (SEM, QUANTA 200F FEG-ESEM, FEI, The Netherlands) and 3D confocal analysis (Leica DCM 3D, Wetzlar, Germany) were used to characterize the morphology and surface characteristics of the scaffolds. Samples were cross-sectioned and gold sputtered prior to analysis with microscope equipments.

The interconnected porosity of the scaffolds was assessed by measuring the volume of water entrapped in the pores of the scaffold using the following equation [31,32]:

$$\text{porosity\%} = [(m_W - m_D) \times \rho_W / V_s] \times 100 \quad (1)$$

where m_W and m_D are the wet (water) and dry masses of the scaffold, ρ_W is the density of the water used as intrusion medium and V_s is the volume of the scaffold determined by a geometrical calculation using a caliper. The weight measurements were carried out on a high precision balance (10⁻⁴ g, CPA225D, Sartorius, Gottingen, Germany). Three measurements were performed for each scaffold type.

Image analysis (Image J[®]) of SEM micrographs was used to assess the mean size (d_M) and size distribution of the pores [31,32]. The pores were analyzed by means of the “particle analysis” tools of the Image J software package, which enables assessing first the area of each pore and then

the pore size with the hypothesis of spherical shape and by correcting the as-obtained values by a factor of $4/\pi$. The textural properties of the scaffolds were studied by low temperature N_2 adsorption-desorption analysis. The tests were carried out at $-196\text{ }^{\circ}\text{C}$ using an ASAP 2000 (Micromeritics, Norcross, USA). Prior to measurements, samples were dried at $35\text{ }^{\circ}\text{C}$ under reduced pressure for 2 days. The specific surface area was determined by the BET (Brunauer-Emmett-Teller) method, while the pore size distribution and mean pore diameter were calculated using the BJH (Barrett-Joyner-Halenda) method.

Compressive mechanical properties were evaluated on wet samples at room temperature on dynamometric machine (Instron 4204) equipped with a 100 N load cell and at a crosshead speed of 1 mm/min. Cylindrical specimens ($d=5\text{ mm}$, $h=10\text{ mm}$) were fabricated according to the ASTM 695/2a standard. The elastic modulus E was calculated by the slope of the stress-strain curve in the strain range of 0-0.1 mm/mm. The study was carried out through analysis of five samples for each type of scaffold.

2.4 In vitro human mesenchymal stem cells culture

Bone-marrow-derived hMSCs (Clonetics, Italy), at the 4th passage, were cultured in 75-cm² flask in α -Modified Eagle's Medium (α -MEM) (BioWittaker, Verviers, Belgium), containing 10% (v/v) FBS, 100 U ml⁻¹ penicillin, and 0.1 mg mL⁻¹ streptomycin (HyClone, Northumberland, UK) at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 . After washing with phosphate-buffered saline (PBS), cells were incubated with trypsin-EDTA (0.25% trypsin, 1mM EDTA, Euroclone) for 5 min at 37°C . Scaffolds for cell culture experiments ($d=5\text{ mm}$, $h=3\text{ mm}$) were first sterilized by soaking in a solution of 70% ethanol and 1% antibiotic (PEN/STREP) for 1 h and subsequently, washed with PBS and pre-wetted in complete medium for 1 h to permit protein absorption and promote material hydrophilicity and then cell adhesion onto the scaffolds.

Cells were seeded with 4×10^4 cells per scaffold, re-suspended in 50 μL of medium. The cell/scaffold constructs were placed in 48-well culture plates and incubated for 2 h to allow for cell

attachment. Subsequently, cell culture medium was added to each well to bring the total well volume to 0.5 mL. The cell/scaffold constructs were cultured for 14 days by changing the cell culture medium every 3-4 days.

Cell adhesion, viability and proliferation were evaluated by using Alamar Blue assay. The cell/scaffold constructs were removed from the culture plates at day 1, 3, 7, and 14, washed with PBS and placed into 48-well culture plates. DMEM (1 mL) medium without Phenol red (HyClone) containing 10% (v/v) Alamar Blue (AbdSerotec Ltd., Oxford, UK) were added to each well and the samples incubated for 4 h at 37°C and 5% CO₂. The solution was subsequently removed from the wells and analyzed by a spectrophotometer (multilabel counter, Victor X3, Perkin Elmer, Milano, Italy) at the wavelengths of 570 and 600 nm. The number of viable cells was assessed by comparing the absorbance values at different culture times with those of the calibration curve. The calibration curve was obtained from the correlation between known cell numbers in the 48 well culture plates with the corresponding absorbance values.

3 Results

Figure 1A reports the DSC curves of the first heating scan of the samples before and after processing by means of TIPS technique. Raw polymers are semi-crystalline, as evidenced by melting peak interval and heat of fusion, which are 110-120 °C and 5.9 J/g for PLC and 120-140 °C and 20.1 J/g for PLA. The PLA processed by means of TIPS evidences a melting range similar to that of the raw material and a slight increase of the heat of fusion up to 23.0 J/g. All of the PLC-based samples processed by means of TIPS technique show a reduction of the melting peak area. This effect is particularly evident in the case of neat PLC copolymer, while for the PLC-90 and PLC-50 samples the melting peak area corresponding to the PLA decreased down to 13.7 and 3.5 J/g, respectively, according to the decrease of the PLA fraction into the blends [35,36]. The non-observance of crystalline peaks on the DSC curves recorded for PLC and PLC-90 samples could be related with the presence of tiny crystallites. Small average crystal size would not appear at the relatively high heating rate used in this work for measurements.

Figure 1B shows the X-ray diffraction pattern of the PLC and PLA samples before and after processing by means of TIPS technique. Raw PLC and raw PLA show their most intense peaks at 2θ values of 16.5°, 18.8° and 22.5°, in agreement with the peaks reported in [37] and related to PLA crystallizing in a pseudo-orthorhombic unit cell. No matching peak has been found in the related PCL co-polymer diffraction pattern (not shown). Surprisingly, PLC and PLC/PLA blend samples processed by means of the TIPS technique have diffraction patterns similar to the patterns observed for raw materials, as an evidence of their crystalline structure.

Figure 2 reports low and high magnification SEM micrographs showing the effect of the composition of the blend on the morphology of the porous scaffolds prepared by means of TIPS. For the prepared samples, the macroporosity was homogeneous through the structure. As shown, the neat PLC scaffold is constituted by aggregated particles and double-scale porosity. Larger pores, of the order of tens of microns, are formed in the inter-particle space. Concomitantly, each particle

shows a nano-size scale fibrous (200-500 μm) and porous morphology. Conversely, the PLA scaffold is characterized by a uniform nano-size scale fibrous and porous morphology, with fibers in the 200-500 nm size range. Blending PLA with PLC turns the morphology of the scaffolds to a uniform interwoven nano-size scale fiber architecture, while the density of the fibers increases with the increase of PLA from 10 (PLC-10) to 50 w% (PLC-50).

Low temperature N_2 adsorption-desorption analysis is used to assess the nano-scale pore structure and textural characteristics of the scaffolds prepared by means of the TIPS process. The results of this test are reported in Figure 3. All of the scaffolds evidence type II N_2 adsorption isotherms (Figure 3A), while the adsorption shifts to high relative pressure values with the increase of PLA concentration. The specific surface area, mean pore size and micropore area of the different scaffolds are shown in Figures 3B and C. In agreement to the morphological observations of the cross-section of the scaffolds of Figure 2, the specific area increases from 7.3 m^2/g for neat PLC up to 148.1 m^2/g for neat PLA. PLC-90 and PLC-50 blend scaffolds have intermediate values of specific surface area, equal to 14.1 and 54.4 m^2/g , respectively. BET analysis also evidences the progressive increase of the specific area of micropores, from 0 up to 23 m^2/g , and the pore size, from 5.6 to 10.5 nm, with the increase of PLA concentration from 0 (PLC scaffold) up to 100% (PLA scaffold).

The preparation of nanofibrous scaffolds with interconnected networks of large pores has been achieved by combining TIPS and NaCl particles leaching techniques. The morphology and pore structure features of resulting scaffolds are shown in Figures 4 and 5. The low magnification SEM images of Figure 4 evidence that the scaffolds are provided of predesigned networks of large pores, with d_M values in the 250 up to 400 μm range. The observance of interconnected macropores in the A pictures of Figure 4 indicated that during the synthesis process the NaCl particles should have contact points, which ensures their successful removal and the formation of throats between adjacent large pores. Nevertheless, the increase of the viscosity of the polymeric solution with the

increase of the concentration of PLA in the starting solution would reduce the NaCl particles sedimentation rate before the TIPS step and consequently, PLC-50 scaffold evidences smaller pore interconnection throats if compared to the other formulations. In the case of PLA sample, the too high polymeric solution viscosity limits the uniform NaCl particles dispersion, not enabling the preparation of porous PLA scaffolds by this combined approach. Blending PLC with PLA also has an important effect on the morphology of the small pores of the scaffolds formed during TIPS step. In particular, the high magnification SEM images reported in Figure 4 show that the PLC and PLC-10 scaffolds are provided of almost rounded pores, with $d_M=15$ and $4\ \mu\text{m}$, respectively. Furthermore, for these scaffolds the presence of globular nano-size scale fibrous structures on the pore walls is observed. The morphology of the PLA-50 scaffold evidences a pore structure characterized by homogeneously distributed nano-size scale fibers, similar to those obtained in the case of TIPS processed PLA-50 and PLA scaffolds and reported in Figure 2.

Confocal analysis images, reported in Figure 5, provides 3D reconstruction of the pore structure of the scaffolds prepared by combining TIPS and NaCl leaching techniques also enabling the assessment of the surface roughness and skewness (defined by ISO 25178 standard). In agreement with SEM results, the scaffolds are provided of interconnected networks of large pores of hundreds of microns in size. Furthermore, with the increase of PLA content a decrease of the roughness of scaffolds surface while the skewness, which is indicative of the symmetry of the topography about the mean plane, is observed becoming closer to 0.

Scaffolds porosity, shown in Figure 6, increases from 85-89% the case of TIPS (-NaCl) scaffolds up to 94-95% in the case of scaffolds prepared by combining TIPS and NaCl leaching techniques (+NaCl). Minor effects are assessed on scaffolds porosity with respect to the different compositions used.

Figure 7 reports the static compression properties of the scaffolds as a function of their composition and fabrication route. The analysis of the σ vs. ϵ curves of the scaffolds, reported in

Figures 7A and B, show that PLC scaffolds are flexible and highly elastic (rubber-like behavior), while the addition of PLA makes the scaffolds more stiff and rigid as confirmed by the increase of elastic modulus. This effect is clearly highlighted in Figures 7C, which indicates an increase of E value from 24 ± 1 kPa for PLC up to 340 ± 60 kPa for PLC-50. The addition of NaCl particles during the fabrication route (+NaCl) results in the decrease of the E values down to 4 ± 0.1 kPa for neat PLC and 42 ± 1 kPa for PLC-50 scaffold. As shown in Figure 7D, similar trends are observed for the $\sigma_{\epsilon=0.5}$, rising the maximum values of 50 ± 3 kPa (-NaCl) and 8 ± 0.1 kPa (+NaCl) in the case of PLC-50 scaffolds.

Figure 8 reports the adhesion efficiency and proliferation trends obtained by the Alamar Blue test performed on the cell-scaffold constructs at different culture times. As shown in Figure 8A, almost 50% of seeded cells adhered to PLC and PLC-90 porous scaffolds prepared by means of TIPS (-NaCl). Lower cell adhesion values, close to 35%, were obtained for PLC and PLC-90 scaffolds prepared by using NaCl particles as porogen agent (+NaCl). The PLC-50 scaffolds (-NaCl and +NaCl) had 30% lower values of cell adhesion. The results of cell proliferation tests shown in Figures 8B and 8C demonstrate that cells grown over 14 days during *in vitro* culture, independently upon blend composition and scaffold architecture. Indeed, the number of viable cells adhered into the PLC scaffold prepared by means of TIPS (-NaCl) increases from $4.1 \pm 0.09 \times 10^4$ at day 1 up to $12.4 \pm 0.12 \times 10^4$ at day 14 (Figure 8B). The addition of PLA to the PLC slightly decreases the number of viable cells into the scaffolds especially at the early time points. Lower numbers of viable hMSCs are obtained for the scaffold prepared by combining TIPS and NaCl leaching techniques (+NaCl).

4 Discussion

TIPS represents one of the most versatile techniques suitable to prepare porous scaffolds composed of polymeric fibers with 100-500 nm size range [11,25,38]. Indeed, TIPS can be applied to a large variety of synthetic and natural polymers, whereas fibers morphology and density can be adjusted by simply changing the polymeric solution composition and the thermal history [25,38]. Furthermore, TIPS can be appropriately combined with other techniques, such as particulate leaching and solid free-form fabrication, in order to improve the size of the pores between fibers for enhancing cell colonization [11,39,40]. The main aim of this study was to blend PLC and PLA polymers applying TIPS technique to enhance the control of the nano-size scale fibrous microarchitectures of the prepared scaffolds, as well as their pore structure and mechanical properties for *in vitro* mesenchymal stem cells culture.

Commercially available PLA polymer and PLC copolymer with a 70:30 L-lactide/ ϵ -caprolactone composition were chosen for processing by means of TIPS from EL-based solutions for the fabrication of stiff, as well as soft and highly flexible porous scaffolds. As shown in Figure 2, and also widely reported in literature [24,25], under appropriate processing conditions TIPS of PLA solution allows fabricating nano-size scale fibrous scaffolds depending on the mechanism of polymer crystallization during solution gelation. In fact, in the case of PLA, it has been demonstrated that the decrease of the temperature of the solution induces the PLA condensation into amorphous nanoparticles followed by its radial crystallization into nano-size scale fibers [24]. Contrarily, the PLC scaffold prepared by means of TIPS shows aggregated microparticles (Figure 2), while its structure is formed by the assembly of nano-size scale fibers. This represents a unique result as, so far, it has been reported that processing PLC solutions by means of TIPS do not enable the fabrication of nano-size scale fibers, because of difficulties in polymer crystallization [41,42]. Hence, in the authors' knowledge, this is the first time that neat PLC nano-size fibrous construct have been prepared by TIPS technique. The results of X-ray analysis evidenced the formation of a

crystalline phase. The absence of melting peak in the recorded DSC seems to support the amorphous nature of neat PLC sample processed by means of TIPS. However, this result can be also attributed to small crystal size. Recently, it has been observed that the nano-size scale structure of PLA processed by means of TIPS technique depends on the concentration of polymer in solution and the quenching temperature [43]. In particular, concentrated polymeric solutions, such as those used in this work, are characterized by polymeric chains intensively entangled to each other and, therefore, nuclei condensation could occur in the proximity of adjacent pre-ordered polymeric chains. In the case of PLA, the high solution viscosity combined with the low quenching temperature selected (-15°C) significantly constrain chains mobility. As a result, PLA crystallization does not follow a preferential direction and the resulting structure is randomly organized (Figure 2). Conversely, the low viscosity of the PLC solution enables chain mobility and crystallization into globular structures resembling polymeric spherulites [43]. These considerations are also in agreement with the results reported in a previous work on the fabrication of porous PCL particles [34] and scaffolds [44] by means of TIPS technique from EL-based solutions. As shown in the results of X-ray analysis, PLC diffraction peaks exclusively correspond to the crystallization of PLA because of the rather low CL molar composition of the PLC used in this work [37]. The addition of PLA to PLC, even at the lowest concentration of 10 wt%, turns the morphology of PLC from globular to an almost random nano-size scale fibrous structure, in agreement to the results reported in [41].

There is a strong correlation among scaffold composition, nano-size scale pore structure and textural characteristics. In particular, if compared to the randomly organized PLA fibers, the globular conformation of the fibers into the PLC scaffold resulted in lower values of adsorbed volume and specific surface (Figure 3). This is probably related to the fact that, the random and highly entangled structure of PLA fibers confers to this sample higher micropore specific surface and overall surface-to-volume ratio [8].

In designing porous scaffolds for TE, one of the most important aspects is the achievement of a porous structure with pores both large enough and sufficiently interconnected to promote cell adhesion and colonization in 3D. In general, scaffolds prepared by TIPS, are characterized by highly dense nano-size scale fiber structures, which are often inadequate for the 3D cell culture [28]. To overcome this limitation, NaCl particles were used in this work as additional porogen during TIPS (Figure 4). The reasons of choosing of NaCl, instead of other more biocompatible material [5,32], are two-fold. First, these particles are insoluble in EL and do not experienced significant chemical and morphological alterations when processed by means of the TIPS technique [44]. Second, the density of NaCl, equals to 2.2 g/cm^3 , is sufficiently high to enable fast particles sedimentation inside the viscous polymeric solution with the consequent formation of an interconnected porosity after their leaching. These considerations are corroborated by the SEM and confocal microscopy results of Figures 4 and 5, respectively, also evidencing that the increased viscosity of the polymeric solution after the addition of PLA to PLC resulted in porous scaffolds with decreased large pore throats. As a result, in the case of neat PLA solution it has not been possible to prepare scaffolds by this approach. However, this limitation can be quite easily overcome by using, for instance, a pre-sintered particulate porogen template followed by the infiltration of the polymeric solution [32].

As expected, the use of NaCl particles as co-porogen results in the increase of scaffolds porosity up to 95% which, in turns, lead to the decrease of scaffolds mechanical response (Figure 6). The porosity, pore size and mechanical response of scaffolds have direct implications on their functionality during biomedical applications. Open porous and interconnected networks are essential for cell nutrition, proliferation and new tissue vascularization [4-6,32]. A porous surface is also necessary for ensuring a mechanical interlocking between the scaffolds and surrounding tissue to improve the mechanical stability of the implant [45]. Polymeric scaffolds with porosity degree in the 60-99% range and pore size between 100 and 500 μm have been used, among others, for bone

and cartilage regeneration [1,3,5,6]. Although higher porosity and pore sizes may facilitate nutrient and oxygen delivery as well as enable more cell adhesion and infiltration, these parameters also affect the mechanical properties of the scaffolds [6]. In this work, the mechanical performance of the prepared scaffolds was assessed in the hydrated state to better simulate the biological conditions. The decrease of mechanical properties with the increase of scaffolds porosity and pore size has been reported for PLA scaffolds prepared by means of particulate leaching and tested in both dry and wet conditions [46,47]. The PLC scaffold is flexible and highly elastic, while its mechanical performance is significantly affected by the relative blend composition and fabrication technique. In particular, in the 0-0.5% strain range, the small pore size and low porosity of PLC scaffold prepared by TIPS resulted in higher stress values compared to PLC scaffolds prepared by means of TIPS and NaCl leaching techniques. Accordingly, E decreased from 24 ± 1 kPa down to 4 ± 0.1 kPa when NaCl particles were used as additional porogen (Figure 7C). Similar results have been reported by Jung and co-workers for PLC scaffold with 80% porosity and prepared by means of the NaCl leaching technique [48]. The results of this work also indicate that the biomechanical properties of the scaffolds can be adjusted depending on the required application by simply blending PLC and PLA in appropriate proportions. The addition of PLA fraction contributes to control the elasticity of the porous scaffold, assuring high rigidity and scaffold stiffness for lower deformation range (until 10%). In particular, PLC scaffolds stiffness can be increased in more than one order of magnitude by the presence of 50% of PLA. The strengthening effect of PLA is also consistent with the results recently reported by Wang and co-workers for PLC/PLA porous scaffolds prepared by TIPS [41].

Scaffolds biocompatibility has been assessed by culturing hMSCs *in vitro* over 14 days. The results of these tests indicate that, if compared to scaffolds prepared by the combined process (+NaCl), those prepared by means of TIPS (-NaCl) enhance cell adhesion. This effect, which is especially evident in the case of neat PLC, can be mainly ascribable to the differences in scaffolds

morphology and pore structure. Indeed, the globular nano-size scale morphology coupled to the lower porosity and smaller pore size of PLC scaffolds prepared without NaCl may result in enhanced retaining and adhesion of cells during static seeding [49]. Conversely, the higher porosity and larger pores size and pore throat of scaffolds prepared by means of TIPS and NaCl leaching combined process may promote the diffusion out of the cell suspension during seeding, finally leading to decreased cell adhesion. Cells have proliferated well on all of the prepared scaffolds. In particular, minor differences are observed with scaffold composition, while slightly lower numbers of viable cells is achieved for porous scaffolds prepared by using NaCl as particulate porogen (Figures 8B and C). Once again, it is important to take into the account that cell proliferation is strongly dependent on the scaffold morphology, porosity, pore size distribution and interconnectivity. Small pores and pore throats act as a separation membrane for cells in suspension and therefore, promote their retaining in the scaffold, especially on the seeding surface [5,49]. Dynamic seeding and culture conditions can be used to improve cell adhesion into the (+NaCl) scaffolds if necessary [50]. The different cell adhesion into the scaffolds can partially explain the observed differences in cell proliferation. Indeed, cell adhered onto the seeding surface are directly exposed to the oxygen and nutrients available in the culture medium and therefore, cell proliferation can be accelerated [5]. In contrast, reduced diffusion of oxygen and nutrients to cells adhered into the scaffold inner regions can slower their proliferation [51]. In summary, all of the scaffolds prepared are biocompatible as they enable cell adhesion and stimulate cell proliferation over the entire culture time investigated.

Conclusions

This work demonstrates that porous scaffolds made of PLC, PLA, as well as blends of these two biocompatible and biodegradable polymers, can be successfully designed and fabricated by TIPS with or without NaCl particles as solid porogen, avoiding the use of harmful solvents.

Most importantly, the proposed approach enables the easy and versatile control of scaffolds nano-size scale fibrous architecture, pore structure and mechanical response. The nano-size scale fibrous morphology and structure strongly depends on the polymeric solution composition and polymer crystallization. In particular, during TIPS process neat PLC co-polymer crystallizes in the form of a globular nano-size scale fibrous structure, while neat PLA and PLC/PLA blends formed uniformly distributed networks of interwoven fibers. Furthermore, the addition of NaCl as particulate porogen during the fabrication route enables the additional formation of interconnected networks of large pores into the scaffolds.

In conclusion, the results of this work demonstrate that scaffolds morphology, texture and mechanical properties can be optimized to promote *in vitro* hMSCs adhesion and proliferation.

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Captions to tables and figures

Table 1: Compositions of the blend scaffolds prepared.

Figure 1: Results of: (A) DSC analysis and (B) X-ray test on the samples before and after TIPS process.

Figure 2: Low and high magnification SEM micrographs showing the morphology of samples prepared by means of thermally induced phase separation (TIPS) process as a function of the composition of the polymeric solution.

Figure 3: Results of samples microporosity obtained from the BET analysis showing: (A) N_2 adsorption isotherms, (B) specific surface, and (C) micropore area and mean pore size of scaffolds prepared by means of TIPS technique as a function of their composition.

Figure 4: Low (A) and high (B, C) magnification SEM micrographs showing the morphology of samples prepared by means of TIPS process combined with NaCl leaching as a function of the composition of the polymeric solution. The insets report the pore size distribution of the macropores taken for pictures labeled A and the mean pore size of the micropores for pictures labeled B.

Figure 5: Confocal 3D reconstructions and resulting roughness and skewness values of the porous structure of samples prepared by means of TIPS process combined with NaCl leaching as a function of the composition of the polymeric solution.

Figure 6: Macroporosity of samples prepared by means of TIPS process with or without NaCl leaching as a function of the composition of the polymeric solution.

Figure 7: Mechanical response under compression of the different PLC scaffolds prepared by means of: (A) TIPS and (B) TIPS combined with NaCl leaching. E (elastic modulus) and σ values at $\varepsilon=0.5$ of the scaffolds as a function of composition and processing technique are given in (C) and (D), respectively.

Figure 8: Mesenchymal stem cells adhesion (A) and proliferation on porous scaffolds prepared by TIPS (B) and by TIPS combined with NaCl leaching (C).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8