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PLLA Scaffold via TIPS for Bone Tissue Engineering

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Tissue engineering offers a promising new approach to repair bone fractures, fractures that do not heal, and fractures due to bone tumors. In this work, two different approaches were tested in order to obtain Poly-L-Lactic Acid (PLLA) porous scaffolds via Thermally Induced Phase Separation (TIPS) for bone tissue engineering application. First, the possibility to produce a composite material, by incorporating Hydroxyapatite (HA) particles in a Poly-L-lactic acid (PLLA) matrix was investigated. Two PLLA/HA weight ratios (70/30 and 50/50) were tested. The results showed that the presence of HA does not influence the phase separation process, i.e. the composite scaffolds microstructure is similar to pure PLLA scaffolds. WAXD analysis confirmed the full incorporation of HA particles into the polymer matrix. Moreover, compression tests showed a fourfold increase of Young module with respect to pure PLLA scaffold. Since the production of scaffolds for bone tissue regeneration is a challenging target, scaffolds must mimic the bone morphology, thus requiring a gradient of pore dimension and morphology along one dimension. To attain this goal, the second part of the work describes the design, set up and test of an experimental apparatus able to set different thermal histories on the two sides of a sample. Scaffolds were produced by following various thermal protocols on both sample surfaces. The results showed that through this technique it is possible to produce scaffolds with a pore size that increases along sample thickness. As a matter of fact, the obtained average pore dimension on one side of the sample was about 70 μm, whereas it was around 240 μm on the opposite surface. By moving along the sample thickness, the pore dimension increased steadily. All things considered, a reliable route for the production of composite PLLA/HA scaffolds with a controlled pore size distribution was assessed, thus offering a valid support to bone tissue engineering.

1. Introduction

The goal of tissue engineering is to assemble functional constructs that restore, maintain, or improve damaged tissues in human organism. The regeneration of tissues is mediated by specific biocompatible and biodegradable 3D device, called scaffolds. These latters require specific characteristics such as: high interconnected porosity, sufficient mechanical strength and appropriate morphology in relation to the tissue to be regenerated (Hutmacher, 2001).
The use of scaffolds for bone regeneration has been widely investigated. Since it was discovered that the bone tissue of mammals contains 58% ceramic Hydroxyapatite (based on dry weight), great efforts have been made to develop phosphate ceramics as a potential implant material (He et al., 2003). Hydroxyapatite has been studied extensively for cell cultures and has been found to possess good osteoconductive properties.
Osteo-articular tissues exhibit a hierarchical structure with a relevant level of complexity; for this reason bone repair and regeneration appears to be a very challenging target. All bones exhibit a compact pore morphology on the outer, so-called compact bone, which confers the necessary strength, and an larger pore morphology on theirs inner, so-called spongy bone, where bone marrow is contained. Therefore, a device thought for bone repair has to present a gradual variation of the pore dimensions along the scaffold thickness to mimic the final desired structure.

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The Thermally Induced Phase Separation (TIPS) is one of the most adaptable techniques to produce porous scaffolds. This technique is based on changes in Gibbs free energy to induce the demixing of a homogeneous polymer solution to obtain a multi-phase system (He et al., 2009). The main operating parameters of the TIPS technique include: polymer properties, solvent to nonsolvent ratio, polymer concentration and temperature vs. time history. With respect to bone morphology, one of the most relevant advantages of the TIPS technique is the ability to control pore size only by varying T vs. time protocol.
In this work, the possibility to produce composite - Poly-L-lactic acid (PLLA) and Hydroxyapatite (HA) - porous scaffolds via TIPS for bone tissue engineering applications was investigated. Two PLLA/HA ratios were tested (70/30 and 50/50 wt/wt) and the as-obtained scaffolds were characterized with various techniques.
Moreover, an experimental apparatus setting different thermal histories on the two sides of a sample was designed, set up and tested, in order to attain a gradient of pore dimension into the scaffold.

2. Experimental

2.1 Materials

Poly-L-lactic acid (PLLA, ResomerTN L 209 S), 1,4 dioxane (Sigma) and double distilled water were respectively utilized as polymer, solvent and nonsolvent to prepare the ternary solution. Hydroxyapatite (HA) was kindly given by Prof. Licciulli from University of Lecce. Granulometric analysis of HA was performed with a laser granulometer Malvern Mastersizer.

2.2 Composite scaffolds preparation and characterization

A homogeneous quaternary solution composed by PLLA, HA, dioxane and water was prepared, with a constant dioxane to water ratio of 87/13 wt/wt. The concentration of PLLA was 4% wt and two different PLLA-HA weight ratios (70/30 and 50/50) were tested. The solution was initially kept at 60 °C, then poured into a cylindrical sample holder. Thereafter, temperature was suddenly lowered to 30°C, by pool immersion in a thermostatic bath, a temperature within the metastable region (Carfì Pavia et al., 2008), for 10 minutes. Finally, a quench by direct pool immersion in an ethyl alcohol bath at a temperature of -25°C was performed. The as-obtained foams were washed in deionised water and dryed at 35°C under vacuum, in order to completely remove any remaining solvent trace. The morphology of the foams obtained was analysed by Scanning Electron Microscopy (SEM) by a SEM-FEI QUANTA 200F on sample cross sections, fractured in liquid nitrogen and gold sputtered (Sputtering Scancoat Six, Edwards) for 120 seconds under argon atmosphere before imaging. SEM images were exported as 24-bit image files using the tagged image file format (tiff) for further analysis. WAXD measurements were carried out by means of a Panalytical X'Pert Powder Diffractometer with 2θ angle ranging from 5° to 70°, with a step angle and a step time of 0.15 degrees and 10 seconds, respectively.

2.3 Scaffolds with a gradient in pore size

In order to obtain a gradient of pore dimension along the thickness of the scaffold, an experimental apparatus able to impose a different T vs time pathway on two sides of a sample was designed and set up. Previous studies about TIPS process have highlighted that, when a nucleation-and-growth mechanism occurs, the pore dimension is mainly dependent by the thermal history (Carfì Pavia et al., 2008). Thus, by varying the residence time in the metastable region, it is possible to control the pore size. The peculiarity of this instrumentation is the possibility to control simultaneously temperature and cooling rate.

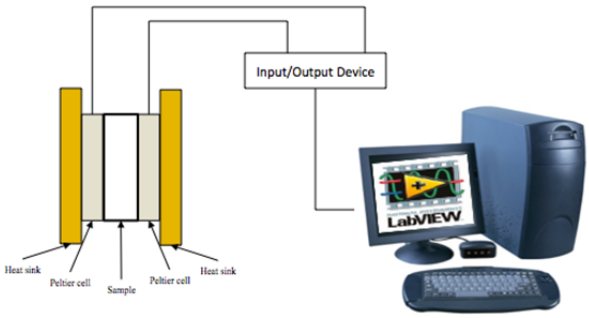

Figure 1: Experimental apparatus.

The experimental apparatus (showed in fig. 1) consists in two Peltier cells, with the related heat sinks, to control temperature on sample surfaces. The sample is shaped as a thick slab, hold in a bag (35x35 mm

surface, 10 mm thickness) made of a multilayer polymeric film (inner layer: polycarbonate, outer layer: polyethylene). The sample holder is inserted between Peltier cells (see Figure 1), and very thin thermocouples were placed on sample holder surfaces, for temperature measurement. All components are connected to an I/O data acquisition device and remotely controlled via personal computer (PC) with a LabVIEW VI (Mannella et al., 2015).

Peltier cells are thermoelectric devices based on Peltier-Seebeck effect, consisting in the conversion of thermal energy to electrical energy and viceversa. Current (I) and heat flow (Q) are directly related by the Peltier coefficient (Π), an intrinsic material property: $Q=\Pi \cdot I$. Moreover, Seebeck (S) and Peltier coefficients are directly related by $\Pi=S\Delta T$. The heat flux globally removed from cold side and transferred to hot side are respectively: $Q_c=\Pi_c I-1/2\ I^2R-K\Delta T$ and $Q_h=\Pi_h I+1/2\ I^2R-K\Delta T$; where R and K are respectively thermal resistance and thermal conductance of the module. It is possible to obtain the total power transferred by subtracting the two heat flows: $P=Q_h-Q_c=SI(T_h-T_c)+I^2R$. The power supplied is then converted into two different heat sources: a thermoelectric heat flux, $SI(T_h-T_c)$; and a Joule's heating, I^2R. Two heat sinks were needed to maintain constant the temperature on one side of each Peltier cell employed.

The electrical current supplied to Peltier cells was controlled via Pulse Width Modulation (PWM) method. By assuming a constant hot side temperature, the temperature difference between the two sides of Pelties cell is:

$$\Delta T = T_h - T_c = \frac{SIT_h - \frac{1}{2}I^2R}{SI+k} = \frac{ST_h\psi - \frac{1}{2}I_M R\psi^2}{S\psi + \frac{k}{I_M}} \quad (1)$$

Where ψ is the "duty cycle" defined by $\psi=I/I_M$, where I_M=5 A is the maximum current supplied (Kraftmakher, 2005). The realized instrumentation is able to control the temperature on two opposite surfaces of the sample by modulating the current supplied to Peltiers.

3. Results and discussion

3.1 Composite PLLA-HA scaffolds

In fig 2a the cross sections of the PLLA-HA scaffold prepared with a PLLA-HA ratio of 70/30 wt/wt are shown. From the micrograph it is possible to appreciate that the phase separation process has correctly taken place, since a porous structure with interconnected pores was observed. Figure 2b shows the same scaffold at a higher magnification: it is easy to observe the HA particles integration into the polymer matrix. The same pore morphology and pore size were found in the scaffolds prepared with a 50/50 PLLA/HA ratio. In this case the presence of HA is more evident (see figure 3a and 3b).

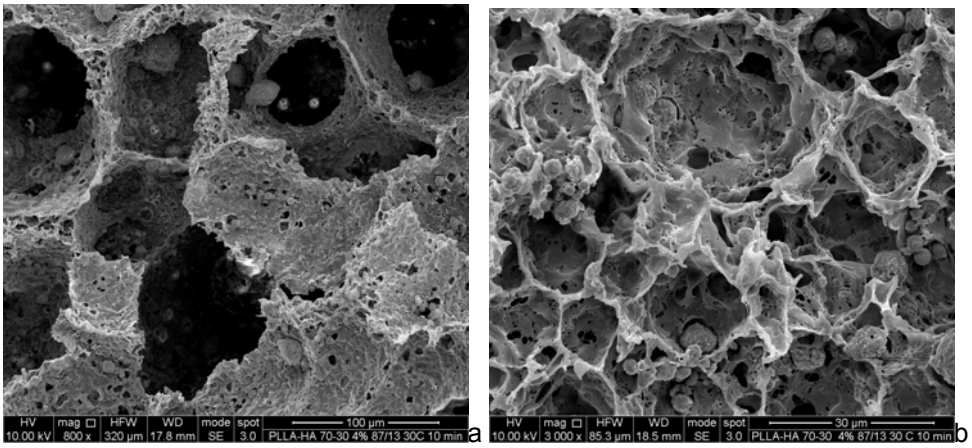

Figure 2: SEM micrographs of the scaffold prepared with a 70/30 PLLA-HA ratio. a) low magnification (800X); b) high magnification (3000X).

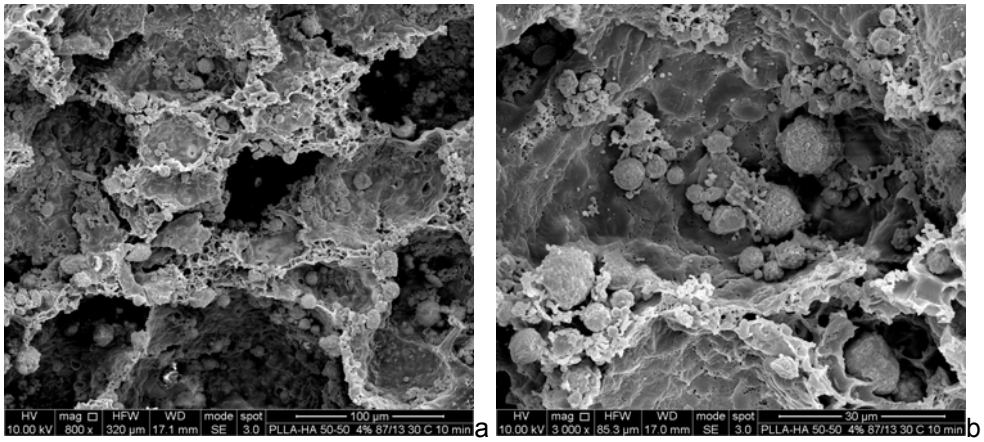

Figure 3: SEM micrographs of the scaffold prepared with a 50/50 PLLA-HA ratio. a) low magnification (800X); b) high magnification(3000X).

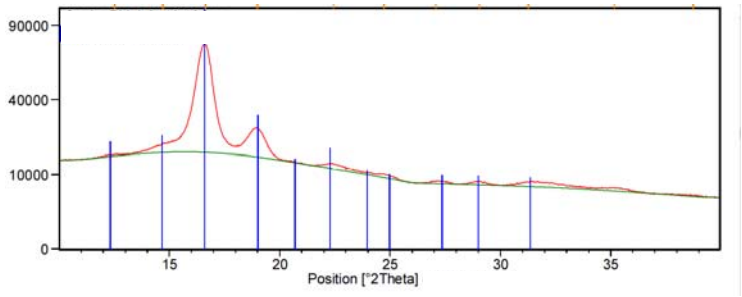

Figure 4: WAXD pattern of a PLLA scaffold.

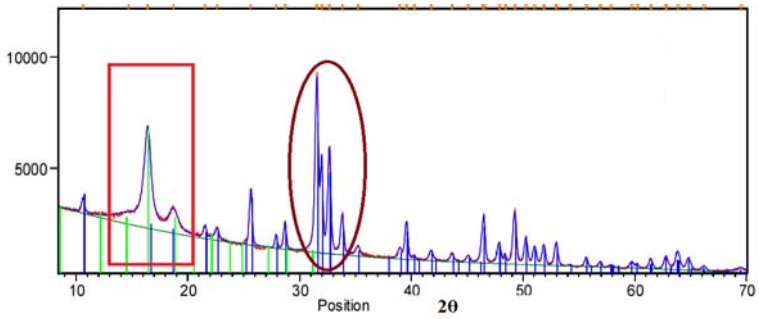

Figure 5: WAXD pattern of a PLLA-HA scaffold.

WAXD analysis confirmed the SEM results. In figure 4 is shown the WAXD pattern of a PLLA foam. It presents two peaks located at 16.5 and 19 degrees, respectively. The WAXD spectrum of PLLA-HA foam (see Figure 5), besides the peaks related to PLLA (highlighted in the rectangular box), has shown a set of peaks associated to Hydroxyapatite (highlighted in the oval box).

3.2 Scaffolds with a pore size gradient

With the experimental apparatus described above, several “symmetrical” scaffolds with the same thermal history on both sample surfaces were prepared, in order to verify the device capabilities and results reproducibility. The solution was kept at 60°C for 10 minutes, then the temperature was lowered at 1°C/min to 35°C (metastable region), and held for 30 minutes. Finally the sample was suddenly cooled to -20°C at the highest cooling rate (25°C/min). The complete thermal history is reported in Figure 6a. Pore size and morphology of foams were analyzed by Scanning Electron Microscopy (SEM) of cross section fractured in liquid nitrogen and gold sputtered before imaging.

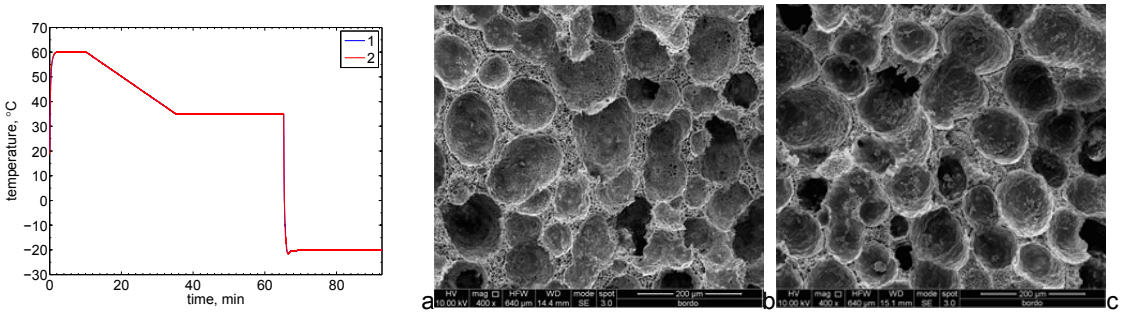

Figure 6: a) Symmetric thermal history; b) Scaffold morphology – Border (Sample 1); c) Scaffold morphology – Border (Sample 2)

With this protocol, most of pores appeared as closed-cells, with a presence of micro-pores on the regions between macro-pores. The results show a good reproducibility, indeed, the scaffold morphology is sufficiently alike between various samples (see Figure 6b and 6c). The residence time in metastable region of the sample center is higher than on the surfaces, by taking into account the time for freezing: as a consequence, pore size is bigger in the sample center rather than on the borders. Macro-pores dimension is about 110-120 μm in the center of sample, and 80-90 μm in the border of sample. Micro-pores dimension is 10-20 μm.

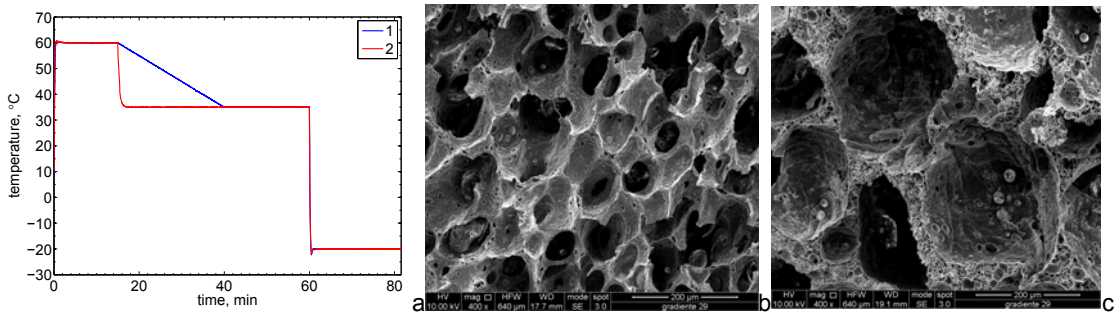

Figure 7: a) Asymmetric thermal history; b) Scaffold morphology – Border 20'; c) Scaffold morphology – Border 45'

Once assured the reproducibility of foam morphology, a procedure to obtain a pore size gradient in the scaffold was researched. A suitable route is defined as follows: after keeping the solution at 60°C for 10 minutes, one side (slow cooling side) was cooled at 1°C/min to 35°C and maintained at that temperature for 20 minutes, whereas the other surface (fast cooling side) was suddenly cooled to 35°C, and held for 45'. Finally, the solution was rapidly quenched to -20°C. A schematization of the process is depicted in Figure 7a, where slow and fast cooling sides are identified by numbers 1 and 2, respectively. From SEM micrographs it was possible to observe that the low cooling side showed an average pore dimension of 70 μm, (figure 7b) whereas in the high cooling side an average pore dimension of 240 μm figure 7c) was obtained.
These results show that by simply varying the T vs time protocol it is possible to achieve a high difference of pore sizes between two borders of scaffold.

4. Conclusions

In this work composite PLLA-HA porous scaffolds were obtained via TIPS. Based on the results, it is possible to state that the presence of the Hydroxyapatite does not influence the phase separation process in terms of demixing temperature. As a matter of fact, a porous and interconnected structure was observed in the bulk of the scaffolds. SEM analysis revealed the presence of HA particles, and their integration in the polymer matrix was confirmed by WAXD analysis. Finally, mechanical tests have highlighted an increase of Young's modulus of composites scaffolds with respect to pure PLLA scaffolds.
A different and complementary approach to produce scaffolds suitable for bone tissue engineering concerned the realization of an experimental technique able to fabricate porous foams with a pore size gradient via TIPS. A pore dimension that varies along the foam thickness was obtained by imposing different thermal histories on the two surfaces of sample. In principle, this procedure can be adopted to produce foams with various overall thicknesses, by varying the distance between the cooling sides (Peltier elements). This type of scaffold

structure has promising applications in tissue engineering wherever a hierarchical architecture involving morphological variations in pore size is required.
By coupling these features, and with the possibility to tune the scaffold morphology, as the TIPS process allows, these devices showed a high potential towards their successful use in bone tissue engineering applications.

Acknowledgments

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