NANOSTRUCTURED POLYMERS FOR SCAFFOLDING APPLICATIONS

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ABSTRACT

Several techniques have been proposed for temporary bone scaffolds production, but they all present various limitations. Indeed, it is very difficult to obtain the coexistence of the micro and nano structural characteristics that are necessary for scaffolding application. In particular, it is important the scaffold nano-structure to assure a good cellular adhesion and growth. The nano-structure is necessary to mime the extracellular matrix that is composed by collagen fibers ranging between 50 – 500 nm, whereas the microstructure has to mime the tissue to be replaced.

We have developed a new supercritical fluid assisted technique for the formation of 3D nano-structured scaffolds, that consists of three sub-processes: formation of a polymeric gel loaded with a solid porogen, drying of the gel using SC-CO_2, washing with water to eliminate the porogen. We obtained 3D Poly(l-lactic acid) scaffolds with elevated porosity (>90%) and a complete interconnectivity, characterized by both a fibrous nanostructure with fibres ranging between 50 and 500 nm and micronic cells, adequate mechanical properties and no-solvent residue. Using our process, it is possible to control both the nanostructure and size and the connection between the micro and the nanostructure. Therefore, the coexistence of the fibrous nano-structure with micronic cells of controllable size has been successfully obtained.

INTRODUCTION

Temporary scaffolds, built using synthetic or natural materials, can be used as surrogates of the extracellular matrix to favor the regeneration of tissue and organs, working as support for cells proliferation[1]. These materials are aimed at eliminating the problems due to donor scarcity, immune rejection and pathogens transfer.

The temporary substitution of different biological materials requires different scaffold characteristics; but, they share a series of common peculiarities that have to be simultaneously obtained: 1. A high regular and reproducible 3-D structure (macrostructure); 2. A porosity exceeding 90% and an open pore geometry that allows cells growth and reorganization; 3. A suitable cell size depending on the specific tissue to be replaced[1-2]; 4. High internal surface areas; the scaffold should present nano-structural surface characteristics that allow cell adhesion, proliferation and differentiation. In particular, a fibrous structure should be formed on the walls of the scaffold to allow cells growth[2]; 5. Mechanical properties to maintain the predesigned tissue structure; 6. Biodegradability, biocompatibility and a proper degradation rate to match the rate of the neo-tissue formation.

Several techniques have been proposed for scaffolds fabrication that include: fiber bonding, solvent casting, particulate leaching, melt moulding, solid free form fabrication, gas foaming and freeze drying combined with particulate leaching[2-3]. Conventional scaffolds fabrication techniques suffer various limitations; particularly, it is very difficult to obtain the coexistence of the macro and micro structural characteristics that have been previously described.

Until now the use of supercritical CO_2 (SC-CO_2) in this field, has been limited to gas-foaming techniques in which it is used as a porogen to produce polymeric foams[4-6]. The process is solventless and very efficient in producing a porous structure; but, generally closed-cells structures are generated during this process.

In this work we propose a different supercritical fluid assisted technique for the formation of scaffolds, that consists of the following sub-processes: 1. Formation of a polymeric gel loaded with a solid porogen. 2. Drying of the gel using SC-CO_2 to form a supercritical mixture with the solvents. 3. Washing with water to eliminate the porogen.

The scaffolds produced using this process should have the following advantages: 1. Accurate reproduction of the shape and 3-D structure of the tissue to be substituted. 2. Controlled and large porosity > 90%. 3. Large internal surface areas. This properties is conferred to the scaffold by the original fibrous nanometric substructure that is characteristic of several polymer gels[7-8]. 4. Very large connectivity at micronic and nanometric levels. 5. Short processing time. 6. Absence of solvent residues.

Therefore, the aim of this work is to study the influence of the process parameters on the formation of Poly(l-lactic acid) (PLLA) scaffolds designed for bone replacement using an ethanol-dioxane PLLA gel loaded with d-fructose particles of selected diameter. Microscopic and mechanical properties of the produced scaffolds will be also characterized.

MATERIALS AND METHODS

Poly(l-lactic acid) (PLLA) with an inherent viscosity ranging between 2.6 and 3.2 dl/g (0.1% in chloroform, 25°C) was purchased from Boehringer Ingelheim (Ingelheim, Germany), d-fructose (m.p. 119–122°C), Dioxane and Ethanol (99.8 % purity) were bought from Sigma Aldrich (S.Louis, MO, USA); CO_2 (99% purity) was purchased form SON (Società Ossigeno Napoli - Italy). All materials were used as received.

PLLA scaffols were prepared following one of the procedures adopted in literature[7]: we prepared solutions with PLLA concentrations of 10%, 12%, 15% w/w in dioxane and added ethanol as the non-solvent with a dioxane/ethanol ratio of 1.7; then we added fructose into the starting solution. The sugar particles were sieved to select the desired size range (250-500, 500-700 μm). The solution was heated at 60°C until it became homogeneous; than, it was poured in steel containers, obtaining samples with a diameter of 2 cm and height of 4 mm. Subsequently, it was placed into a freezer at -18°C for 1 hour to obtain the gel. Then, the gel was dried using SC-CO_2[9]. The supercritical CO_2 extraction of the solvent from the polymeric gel lasted 4 hours. A depressurization time of 10 minutes was used to bring back the system at atmospheric pressure. After the supercritical drying, the obtained structures were poured in distilled water for 24 hours to eliminate the fructose. Subsequently, the scaffolds were put in a oven for 4 hours at 40°C, to evaporate water.

RESULTS AND DISCUSSION

An aerogel is a polymeric structure characterized by a very large porosity and can be formed by a network of fibres with nanometric diameters. Two major problems in aerogel production are to find the right formulation of the solvents and of the process to induce the “gelation” and to eliminate the solvents from the structure avoiding its collapse: evaporation of the solvent produces the destruction of the nanostructure due to the force exerted by the surface tension of the evaporating liquid. Supercritical CO_2 gel extraction is a new process that allows the drying of polymeric gels through the formation of a supercritical solution between CO_2 and the liquid solvent. The supercritical mixture shows no surface tension and can be easily eliminated in a single step by the continuous flushing of SC-CO_2 in the drying vessel[9]. Aerogel structures can possess some of the nanometric characteristics useful to produce scaffolds, but large interconnected cavities are generally missing.

Then, we evaluated the possibility of superimposing a microporous structure in the aerogel to obtain scaffolds. We proposed an hybridization of the supercritical drying process with the particulate leaching[10-11]. An alternative to produce large cells inside a polymeric matrix is represented by the use, as porogen, of an insoluble compound that in our process has to be introduced into the polymeric solution before the gelation. We selected d-fructose particles of controlled diameter as the porogen, since it has a large solubility in water, it is insoluble in SC-CO_2 and has been already successfully used as porogen. The introduction of a large quantity of solid in the liquid solution before gelation can strongly interfere with the gelation process itself. Therefore, we performed several tests on PLLA gels with different concentrations of polymer: 10%, 12% and 15% w/w and adding fructose particles with

diameters ranging between 250-500 μm and 500-700 μm. Gelation and supercritical drying were successful[12]. Moreover, we also performed an analysis of the grade of interconnection of the material and on the surface area by N_2 adsorption-desorption. We verified that all samples presented a completely interconnected structure (i.e., a grade of interconnection of about 100%) and large surface areas (i.e., about 45 m^2/g).

In figure 1 we report SEM images related to the scaffolds formed by PLLA + porogen obtained using different loadings and particles diameters. A very regular structure is obtained, characterized by a large cellular structure due to the porogen, embedded into a network of nanometric fibers that represent the original gel structure.

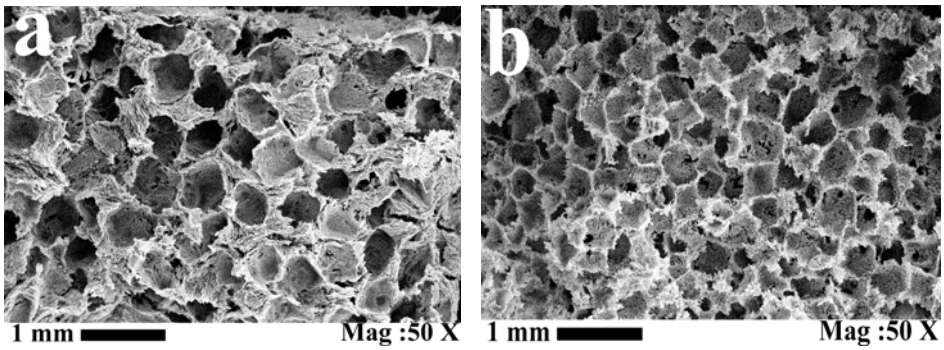

Figure 1: SEM images of PLLA scaffolds obtained by supercritical gel drying combined with particulate leaching. a) scaffold obtained using 500-700 μm d-fructose particles; b) scaffold obtained using 250-500 μm d-fructose particles.

A SEM image produced at large magnification is reported in figure 2, in which image, it is possible to observe that the fibrous sub-structure characterizes not only the polymeric network, but also the cells wall. In particular, fibers size ranges between 50 and 200 nm add further porosity and interconnectivity and produces the walls "roughness" that should be the key factor for cellular growth.

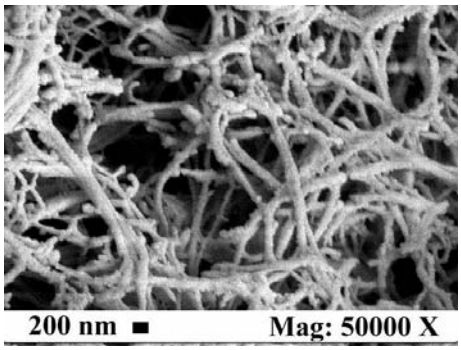

Figure 2: SEM images of an enlargement of part of a cavity

We also measured the compressive modulus of some of the scaffolds obtained to verify if mechanical resistance was compatible with the one required for scaffolding application. Indeed, to be used as a support matrix, the mechanical strength of the scaffold is essential for cell culture and ultimate organ formation; as usual, 100 kPa are necessary for bone scaffolds application. The 12% and 15% w/w scaffolds obtained using fructose particles of 250-500 μm showed a higher compressive modulus with respect to the same scaffolds obtained using the porogen of 500-700 μm. In particular, the mean compressive modulus of 15% w/w scaffolds obtained with 250-500 μm fructose particle size increases up to 81 kPa.

Subsequently, we focused our attention on the inclusion of hydroxyapatite particles inside the polymeric structure with the aim of modifying the scaffold resistance and improving the formation of an environment suitable for bone cells growth; indeed, hydroxyapatite forms more than 50% of the

natural bones and can behave as a support for the structure, increasing the compressive modulus. It also allows to create an environment that better mimes the extra-cellular matrix.

We loaded hydroxyapatite nanoparticles (< 200 nm, Sigma Aldrich) inside the starting solution before the gelation and we analyzed the scaffolds obtained by SEM and EDX. As it is possible to observe from the EDX analysis reported in figure 3a-b, hydroxyapatite nanoparticles are completely dispersed inside the scaffold structure; indeed, the typical element of hydroxyapatite (calcium) is uniformly distributed along the whole section of the scaffold (see figure 3b).

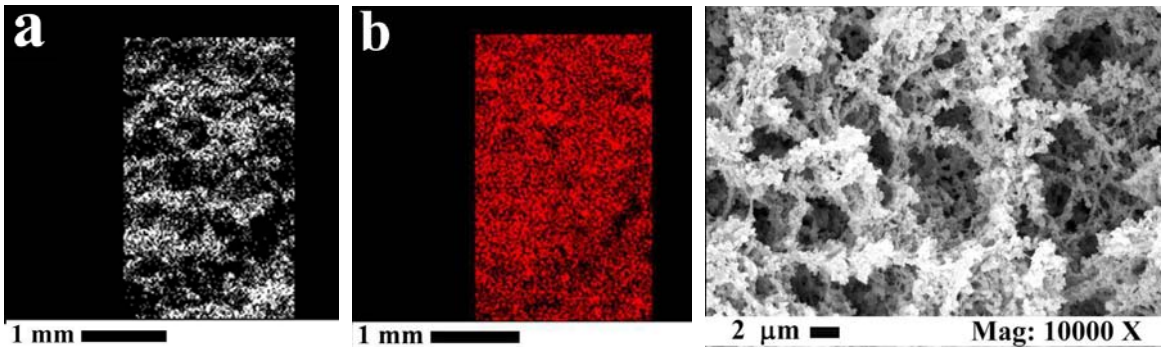

Figure 3: EDX images of a hydroxyapatite loaded PLLA scaffold: a) carbon distribution; b) calcium distribution. SEM image of a hydroxyapatite loaded scaffold cells wall (c).

Moreover, as it is possible to observe from SEM image reported in figure 3c, a modification of scaffold morphology is evident if compared to unloaded PLLA scaffold in figure 2. Hydroxyapatite nanoparticles appears on the surface of polymeric fibers of cells wall forming a hybrid particle-fiber crosslinked structure.

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