Simvastatin-Loaded Poly(ε-caprolactone)/Poloxamines Osteogenic Biocompatible Porous Blends Prepared by $scCO_2$ Foaming/Mixing

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Abstract: Polycaprolactone (PCL)/poloxamines (PLXs) porous blends were prepared using a supercritical carbon dioxide-assisted foaming/mixing (SFM) process and aiming to prepare cytocompatible porous biomaterials with tunable bioerosion rates, bioactive molecules release and osteogenic features. Two different PLXs were employed: T908 and T1107. Pure PCL, pure PLXs and three distinct PCL:PLXs blends (75:25, 50:50, 25:75 %w/w) were processed at constant pressure and temperature conditions. Simvastatin (an osteogenic and angiogenic bioactive molecule) was incorporated into PCL and into PCL/PLX blends using the same SFM methodology and processing conditions. All obtained biomaterials were physically, thermally, chemically and biologically characterized applying a wide range of techniques and in vitro methods. Results indicated that employed PLXs are miscible with PCL for all tested compositions. Prepared porous PCL:PLX biomaterials eroded rapidly in the first week (due to PLXs dissolution) and went through slow and almost constant erosion rates in the subsequent weeks (due to PCL hydrolytic degradation) in phosphate buffer pH 7.4 , which explains the observed simvastatin rapid initial release and its subsequent sustained release for longer periods. PCL and PCL:PLX 75:25 %w/w porous biomaterials showed a high cytocompatibility with SAOS-2 and no cytocompatibility differences were observed between simvastatin-loaded and non-loaded samples. In addition, prepared biomaterials also promoted mesenchymal stem cells proliferation and their differentiation into osteoblasts. Results demonstrated the feasibility of using the SFM method for the development of simvastatin-loaded PCL/PLX osteogenic and biocompatible porous blends presenting distinguishable erosion and release profiles, cycompatibility and potentially advantageous osteogenic features for bone fractures treatment.

Keywords: Poly(ε-caprolactone)/poloxamines porous blends, supercritical carbon dioxide assisted foaming/mixing, simvastatin loading/release, cytocompatibility, mesenchymal stem cells proliferation/differentiation.

INTRODUCTION

Many techniques allow the conventional production of porous scaffolds, however when the incorporation of bioactive additives is intended, these methods make use of organic solvents/chemicals, which must be removed by additional extraction/purification steps, and/or use high temperatures that may induce drug thermal/chemical degradation [1-4].

Supercritical fluids (SCF), namely supercritical carbon dioxide ($scCO_2$), have emerged as "green" routes to overcome the drawbacks of conventional techniques [1, 5]. Adequate

porosity and pore sizes may be created controlling process parameters. Upon depressurization, when the polymer phase is supersaturated with CO_2, thermodynamic instability occurs, and the gas leaves the polymer phase occurring gas bubble nucleation. This nucleation stage involves the assembling of CO_2 molecules, with concurrent homogeneous and heterogeneous nucleation mechanisms occurring upon phase separation of the polymer–CO_2 solution. Moreover, the use of $scCO_2$ as mobile phase can facilitate the diffusion of an additive and improve its incorporation rate in inorganic matrixes, and in previously processed polymeric or composites material, as well as simultaneous preparation of 3D structures and the incorporation of additives at mild processing conditions [6-7], avoiding several steps in the production of these materials that characterize conventional techniques.

The objective of this work was to develop 3D Poly-ε-caprolactone:Poloxamine (PCL:PLX) porous biomaterials that exhibit chemical, mechanical, morphological and biological properties suitable for hard tissue regeneration, and that may be used alone or incorporated into injectable hydrogels and fast-setting bone/dental cements. PCL and PLX (Tetronic® 908 and Tetronic® 1107) were processed at different weight ratios, with and without simvastatin, by means of SFM. To the best our knowledge, this is the first time that scaffolds of PLX and its mixtures with simvastatin and PCL were prepared using supercritical fluids. Microstructure and erosion rate of the samples, simvastatin release profiles, cytocompatibility and in vitro promotion of alkaline phosphate activity were evaluated in detail.

MATERIALS AND METHODS

Chemicals: PCL (Mn 42500 g/mol) was from Sigma-Aldrich (St. Louis, MO, USA) and used as powder [7]. Poloxamines Tetronic® 908 (T908; Mn 25000 g/mol) and Tetronic® 1107 (T1107, Mn 15000 g/mol) were from BASF (New Milford, CT, USA). T908 was hand grinded before use. Simvastatin (98% purity, Mw=418.57 g/mol) was from AK Scientific Inc. (Union City, USA).

Methods: PCL:T908 and PCL:T1107 0:100, 25:75, 50:50, 75:25, and 100:0 w/w (total weight 1.3g) were physically mixed in 10 ml glass vials with inner diameter 1.7 cm, with and without simvastatin (0.01% w/w=0.1 mg/g) and were placed in a high-pressure cell at a constant temperature. CO_2 entered the high-pressure cell up to the desired pressure of 20 MPa. Two temperatures were tested, 40ºC and 43ºC, according to melting behavior of each PLX; namely, T1107 melted at moderated pressure and temperature conditions (approximately 10 MPa and 40ºC) whereas T908 needed higher processing parameters (approximately 20 MPa and 43ºC). The system was kept at constant pressure and temperature for 1 h, under magnetic stirring (700 rpm) to homogenize the supercritical phase. Then, depressurization was performed at 1 MPa/min. The obtained PCL:PLX samples were kept in vials protected from light.

CHARACTERZATION

Infrared spectra were obtained using an Infrared Spectrum Jasco 4200 type A, in the wavenumber range of 500 to 4000 cm^{-1}, with 4 cm^{-1} of resolution and 256 scans. The spectra were analyzed using Jasco Spectra analysis software.

Simultaneous Data Thermal (SDT) equipment (TA Q100) was used to determine in duplicate the overall thermal events (melting temperature, degradation temperature, weight loss) of the processed samples (7-11 mg) between 25 and 600 ºC at a heating rate of 10 ºC/ min, using the software TA Universal Analysis.

Tg values of PCL, PLX and each sample were determined by means of DMTA-pocket technique using a Tritec 2000 DMA (Triton Technology Ltd., UK). Duplicate samples were

analyzed applying three frequencies (1, 5 and 10 Hz) in the temperature range of -150 ºC to +20 ºC, using a heating ramp of 10 ºC/min.
Samples were freeze fractured and scanning electronic microscopy (SEM) images were taken using an EVO LS15 Zeiss equipment in the variable pressure mode.
Average pore diameter, pore volume and surface area were determined by nitrogen adsorption using an ASAP 2000 Micromeritics. Surface area was determined by the BET method, pore volume and average pore diameter were calculated using BJH method. The density of the samples was measured by helium picnometry (Quanta-Chrome, MPY-2). The results are the average and standard deviation of two samples.
The hydrolytic degradation assay was carried out in triplicate for 4 months. Samples were weighed (~50 mg of initial weight) and pretreated with 5 M NaOH (5 ml) at room temperature for 72 h [8]. After that period, the medium was replaced by phosphate buffer pH 7.4 (5 ml) at 37 °C. Once a week the materials were removed, rinsed with water, dried at 37 °C for 12 h, weighed and again immersed in the buffer.
For the simvastatin release, PCL and PCL:PLX samples were immersed in 2.0 ml of phosphate buffer pH 7.4, and kept at 37ºC and 100 rpm. At pre-established intervals of time, 500 µl were withdrawn and replaced with the same volume of fresh buffer at 37 ºC. Simvastatin released was quantified (total amount and relative content in lactone/hydroxy acid forms) using a LiChroCART RP-C18 (3.9 mm x150 mm, 5 µM) column kept at 25 ºC, an UV–Vis diode array detector (238 nm, L-4500 Merck–Hitachi, Germany), and acetonitrile:28 mM phosphate buffer pH 4 65:35 vol/vol as isocratic mobile phase at 1 mL/min flow [9].
Cytotoxicity (LDH Cytotoxicity Detection KitPLUS, Roche, Spain) was evaluated in duplicate using SAOS-2 human osteogenic sarcoma cells (HTB-85, LGD Standards, ATCC, Manassas, VA) according to the direct contact test ISO 10993-5:1999 standard. Slabs (0.9x0.2x0.2 cm, 15-30 mg) with and without drug were UV-irradiated for 30 min and then placed in 24-well plates with 200,000 cells per well. Culture medium, cells in culture medium, and cells in culture medium with lysis factor were used as blank, negative, and positive controls, respectively.
For proliferation and differentiation studies PCL:PLX 100:0 and 75:25 slabs (30-50 mg) with an without simvastatin were sterilized by UV-irradiation and placed in 24-well plates. Mesenchymal stem cells (StemPRO® human adipose derived stem cells from Gibco Invitrogen) were maintained in MesenPRO RS™ (Gibco Invitrogen) medium with 2% growth supplement, 1% glutamine and 1% penicillin/streptomycin and then seeded (60,000 cells per well, 2.0 ml) over the samples and simultaneously in the upper compartment of cell culture inserts. Confocal microscopy images were taken at several times in order to monitor cell viability on the samples. Cells seeded over PCL samples in osteogenic differentiation medium and in medium containing simvastatin (dissolved in a concentration similar to that the implants would provide to the medium) were used as positive controls. The experiments were carried out in triplicate. Cell proliferation (using the Pico Green dsDNA quantification protocol) and alkaline phosphatase (ALP) activity were monitored at 3, 7 and 14 days. The absorbance was recorded at 405 nm and the ALP content determined using a p-nitrophenylphosphate calibration curve. The values were normalized to the protein content as estimated by BCA assay. DNA quantification was carried out using the Quant-iT™ Pico-Green dsDNA assay kit (Invitrogen™,Molecular Probes™, Oregon, USA). The amount of DNA in each sample was calculated using a standard curve relating quantity of DNA and fluorescence intensity.

RESULTS

Processed PCL and T908 had similar melting (T_m) and degradation (T_d) temperatures, while T1107 exhibited lower values (~54 ºC and ~390 ºC, respectively).The polymers mixtures presented decreasing melting and degradation temperatures with decreasing PCL quantity (Figure 1A). All samples regardless the PLX type kept their glass transition temperature between that of pure PCL and that of pure PLX (Figure 1B). A single combined loss tangent maximum was observed in the DMTA runs, which corroborated that the samples are made of miscible components (Jones et al 2012). Thermogravimetric analysis shows that all PCL, PLX and PCL:PLX samples present a corresponding mass loss of nearly 100 % of the total mass. Additionally, FTIR-ATR reveals the presence of PLX and PCL in the final formulations (not shown).

However, melting and degradation temperatures obtained are above the physiological temperature, making these polymers and blends suitable for biomedical applications.

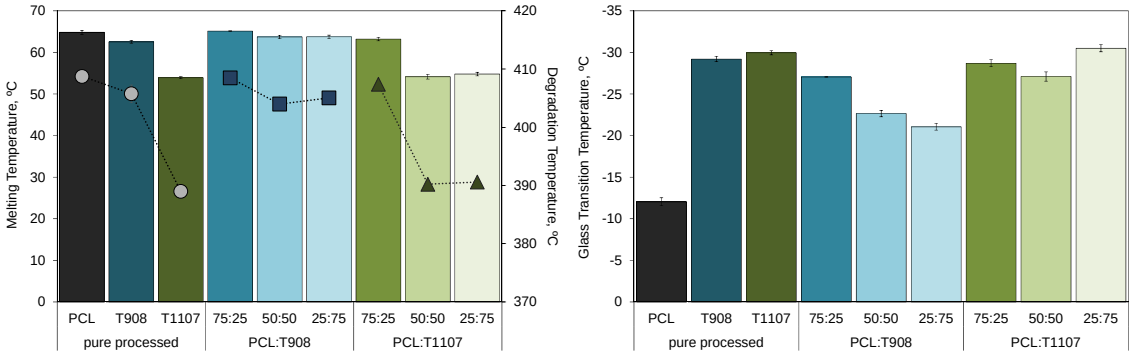

Figure 1 – Melting (columns) and degradation temperature (symbols) (A) and glass transition temperature (B) of pure processed polymers and their blends.

True density values of PCL and PLX were approx. 1.0-1.2 g/cm^3 which is in agreement with the values reported in literature for polymers and also with suppliers' information [7, 10, 11]. Simvastatin did not cause significant changes in the samples density, as expected from its low proportion (0.01 wt%).

Materials surface area ranged between 0.4 and 0.7 m^2/g, where the lowest values matched those samples with the greatest PCL quantity. Pore volume and average pore diameter determined from nitrogen adsorption were quite analogous between the different samples, with and without drug. Although the effect of PLX type was not clear, PLX quantities above 50% tended to slightly improve the porosity and the surface area. Overall, the implants had low porosity in the range of nitrogen adsorption technique. The influence, although quantitatively small, of the PLX proportion on the samples porosity findings may be attributed to specific interactions between CO_2 and polymers functional groups, differences in the length of the polymers, and increased gas dissolution in the amorphous regions of polymers [12-13].

It can be observed by SEM pictures (Figures 2) that different PLX proportions originated distinct porous morphologies. In general, samples containing PLX presented irregular pores in a wide range of sizes, including very small pores within the walls of larger ones; no major differences were observed between the two types of PLX. As PCL proportion increased, the pores apparently became ordered and larger. Apparently interconnected pores were observed in all samples which is particularly relevant for hard tissue regeneration: scaffolds should contain interconnected macropores (between 200-900 µm and between 1.2-2.0 mm) for cell/nutrient diffusion and vascularization, as well as micropores and mesopores (smaller than

2 nm and between 2-50 nm, respectively) for faster degradability and efficient loading, transport and release of bioactive substances [14-15].

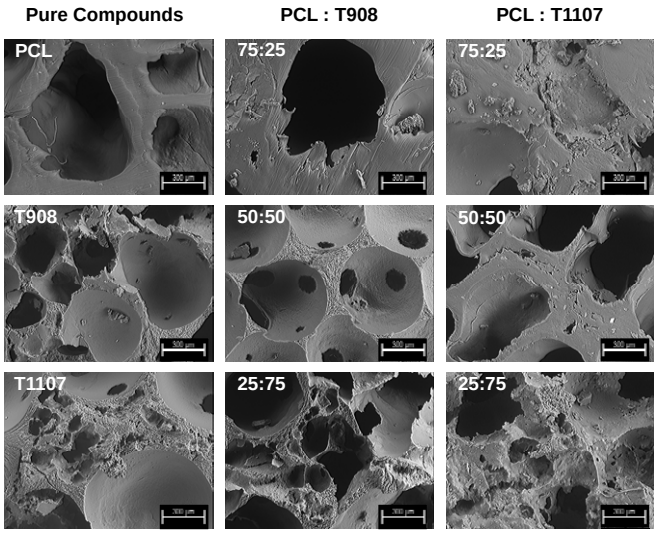

Figure 2 - SEM micrographs of PCL, PLX and PCL:PLX blends processed by supercritical CO2 assisted foaming/mixing. Scale bar 300 μm.

Hydrolytic degradation assay showed that 100% PCL lost less than 1% weight after 2 months. Samples containing PLX showed a rapid weight decrease in the first week followed by a slower weight loss rate until the end of the study. The weight loss percentage in 2 months was somehow lower than the initial percentage of PLX in the samples, which may be explained by the entrapment of PLX into non-eroded PCL matrix.

Simvastatin release from the polymers and polymeric blends presented values higher than expected taking into consideration the weight loss for the same period of time. Even though, the concentration in the release medium was in the reported range to induce differentiation (reported to be 1 μM in culture medium [9]). This may be attributed to fast release of the drug deposited on/near the surface of the material and also to an increase in the porosity due to PLX dissolution. Nonetheless the released quantities were congruent with the relative hydrophilicity of each sample.

Samples of 100% PCL and PCL:PLX 75:25 w/w showed high cytocompatibility with osteoblasts, and no differences were observed between samples containing or not simvastatin.

Mesenchymal stem cells (MSC) were seeded over the implants for 3, 7 and 14 days and their viability and proliferation over the implants was monitored. Confocal microscopy images and dsDNA allowed to observe the strong influence of sample composition on cell growth (Figure 3). Both PCL and PCL:T908 75:25 w/w seemed to be good substrates for the direct culture, assisting adhesion and proliferation. Conversely, PCL:T1107 75:25 w/w samples were ineffective to provide a good environment for cell culture, which may be related to the density of PEO chains on the surface, as previously reported for some PLX grades [16-18].

Simultaneously, MSC were cultured in the presence of PCL and PCL:PLX 75:25 w/w samples placed in the inserts. During the first week, the PLXs and simvastatin hindered the cell proliferation, contrarily to PCL samples with and without simvastatin. After 14 days of culture, PCL (with and without drug) and unloaded PCL:PLX samples assisted proliferation. However, the inferior cell proliferation observed for PCL:T1107 75:25 w/w could be related with known cellular biochemical stress caused by osteogenisis. Thus, the ALP activity was determined and PCL:T1107 75:25 w/w blends (with and without drug) induced a temporal

reduction of the ALP expression. Although simvastatin did not affect SAOS-2 viability and kept its active form during the drug release assay, the quantity released from the blends was deleterious for MSC survival and differentiation into osteoblasts. In addition, after 3 and 7 days of cell culture PCL and PCL:PLX blends induced cell differentiation similar or higher than the positive control. PCL solely combined high levels of proliferation and differentiation for all time points, especially at day 14 were it was possible to observe a stabilization of the cell proliferation and remarkable increase of ALP activity for PCL-simv relatively to the positive control (Figure 4).

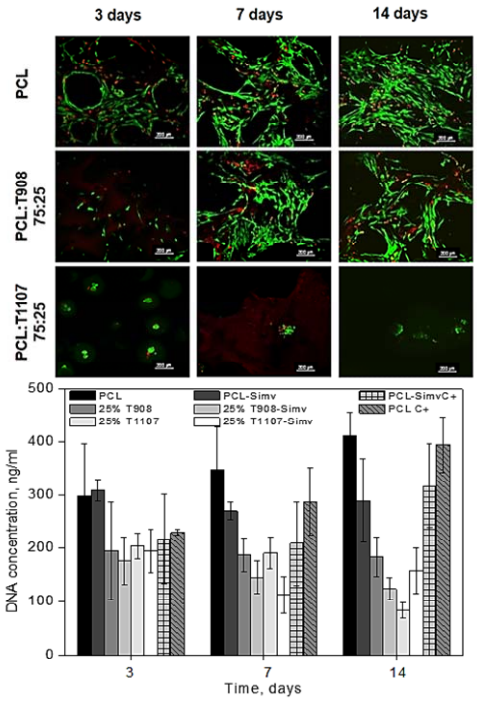

Figure 3 - Confocal microscopy images (dead/alive assay) of MSC seeded over samples (A). Time evolution of proliferation of MSC directly cultured on samples (B).

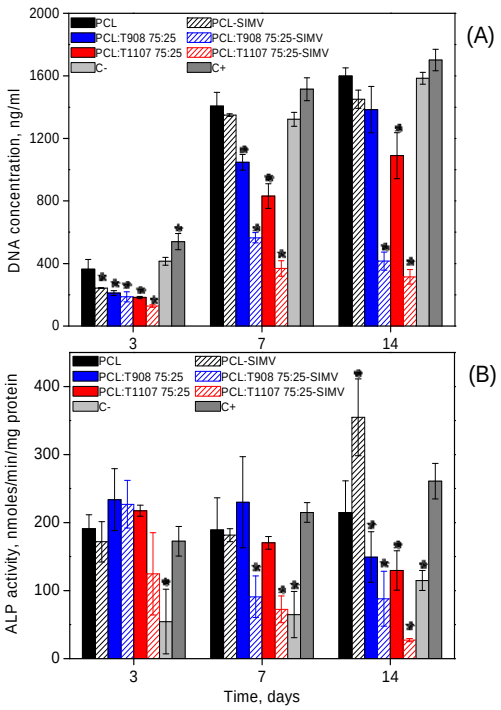

Figure 4 - Time evolution of proliferation (A) and differentiation of MSC cultured in the presence of samples placed in the inserts (B).

CONCLUSIONS

The realization of this work presupposed the fabrication of PCL, PLX and PCL:PLX scaffolds loaded with simvastatin through supercritical fluids, a green technology process. The main goals were attained once PCL:PLX biodegradable scaffolds were produced and successfully loaded with simvastatin, several proportions were studied, distinct release profiles and distinct cellular responses were obtained. Thus, chemically the pure compounds and the blends presented no alteration induced by processing with $scCO_2$. Also, PCL, PLX and blends showed thermic characteristic that allow them to be used in the physiological media. Pore sizes and porosities were found to be appropriate for hard tissue engineering application. Regarding in vitro hydrolytic degradability, it was found that the weight loss was in agreement with the relative quantities of PLX incorporated in the blend. PCL and PCL:PLX 75:25 w/w systems presented a sustained release profile, and the higher quantities of simvastatin released was obtained for the blends. Although the MSC proliferation and differentiation was distressed by the PLX-simvastatin combination, the main purpose of this

work was accomplished. Supercritical CO_2 was successfully and easily used to create simvastatin-loaded systems based on PCL and poloxamines with distinct release profiles and in vitro biological activity. Additionally, $scCO_2$ technology allows different routes for industrial processing, such as extrusion and injection molding, which will in due course define the application.

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