BIOMATERIALS DESIGN AND PROCESSING AVOIDING TOXIC CHEMICALS

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ABSTRACT

The work proposed herein describes the design and fabrication of biomedical devices with controlled biochemical and biophysical properties by means of toxic-free processes. In particular, we developed novel approaches involving the use of green compounds for obtaining biomedical devices in form of porous microparticles suitable as cell carriers, biofilms with tailored morphological and structural properties, as well as three dimensional porous scaffolds for cell culture and new tissue regeneration. The developed processes are based on the use of green compounds, namely supercritical CO2 and biocompatible solvents, such as ethyl lactate and ethyl acetate. Biocompatible and biodegradable polyestersnamely polycaprolactone, polyethylene oxide, polylactic acid and poly(lactide/caprolactone) co-polymer have been selected as main components for the development of biomedical devices. Concomitantly, gas foaming, phase separation and porogen leaching techniques have been investigated for biomaterials processing and the operating conditions optimized in order to achieve a fine tune over the final properties of the biomedical devices.
The results of this study demonstrate that by the appropriate selection of materials and processes it has been possible the design and fabrication of biocompatible and biodegradable platforms, such as porous microparticles, biofilms and porous scaffolds, with controlled morphologies and desired nano-metric, micro-metric and macroscopic structural features. The as obtained devices have also demonstrated to be biocompatible and suitable for applications such as cell delivery and tissue engineering.

INTRODUCTION

To date, the pursuit of biocompatible and biodegradable materials characterized by a three dimensional nano- and micro-scale structure represents a new realm of matter of current research in functional biomaterials design and fabrication. Owning a high porosity and a large surface area, nano and micrometre-scale materials offers outstanding properties in terms of the flexibility of surface functionalities, the control of transport properties and mechanical performance compared with any other known material structure [1].
Open-pore biodegradable microparticles are object of considerable interest for biomedical applications, particularly as cell and drug delivery carriers in tissue engineering and health care treatments [2]. Porous microparticles for drug delivery are a powerful tool in applications were either low or high molecular weight molecules, such as growth factors and proteins, must be released in a specific area at programmed rates and times. Porous biocompatible and biodegradable microparticles are also used as platforms for cell seeding, expansion and culture.
The possibility to design and fabricate 2D platforms characterized by well controlled morphology, topography and architecture may be of great interest in the biomedical field especially to study basic cell behaviour [3]. Based on the use of fabrication techniques developed by the silicon microtechnology industry, several studies were reported about the

correlation among various micro- and nano-features such as lines, wells, holes and more with cell orientation, migration, morphology and proliferation.
Porous 3D scaffolds with a nanoscale fibre structure possess unique transport, structural and biophysical properties for technological and biomedical applications [4]. Indeed nanofibrous scaffolds are able to mimic the collagen structure of the native extracellular matrix, enhancing cell/material cross-talking at the interface and promoting cell adhesion, proliferation and differentiation.
There are different techniques which can be used to obtain micro- and nano-scale biomaterials with controlled biophysical and biochemical properties.
Phase separation is one of the most interesting approaches to fabricate nanofibrous materials with controlled morphology and structural properties by a highly versatile and large-scale approach. [4,5]. Polymeric scaffolds can be obtained by phase separation caused by antisolvent addition, using either conventional liquids or by employing supercritical fluids [5,6]. Phase separation from a polymer-solvent solution is based on the thermodynamic de-mixing of the system into a polymer-rich and a polymer-poor phases, for instance, by cooling down the solution below a binodal solubility curve [5]. This approach, named thermally induced phase separation (TIPS), allows for the large-scale formation of nanometre-scale fibrous structures with characteristic diameter in the range of 50-500 nm by selecting the appropriate polymer/solvent combination and controlling cooling temperature and the kinetics [4]. TIPS can be also used to fabricate porous microparticles from biocompatible and biodegradable materials [7].
Gas foaming is another powerful approach to produce porous biocompatible and biodegradable materials for biomedical applications [8,9]. The physical and chemical structure of biodegradable polymeric foams can be fine tuned by the appropriate materials selection and by the optimization of the operating parameters. As a direct consequence, polymeric foams can be able to fulfil the desired performance for specific tissue engineering applications, such as scaffolds for soft and hard tissue repair/regeneration [8].
Gas foaming is a two steps process. In the first step, the polymer is saturated with the blowing agent, which is typically supercritical CO_2 ($scCO_2$), at high pressure and at a well definite temperature. Once saturation is completed, a controlled pressure quench provokes the formation of a supersaturated $scCO_2$/polymer solution and the nucleation and growth of pores inside the polymeric matrix.
Foaming of polymers with $scCO_2$ is highly advantageous in terms of scaffolds design and manufacturing. Indeed, sorption of even small amounts of CO_2 by polymers results in substantial changes in their physical properties, which dictate their processing and foaming characteristics. The important physical properties include viscosity, permeability, interfacial tension, and glass transition temperature [9]. Consequently, the optimization of the $scCO_2$ foaming process may open a wide range of opportunities for designing novel multi-functional materials and products for both industry and research fields. Furthermore, $scCO_2$ is non-toxic and may allow for the set up of clean fabrication routes for biomaterials processing fabrication, avoiding the use of toxic compounds potentially harmful to cells and biological tissues.
In this work we report a comprehensive study of novel and clean approaches for the design and fabrication of different platforms with controlled micro- and nano-structural properties for biomedical applications. In particular, to overcome the limitations related to the use of toxic solvents, in this work we investigated the use of $scCO_2$ and ethyl lactate (EL) and ethyl acetate (EA) for processing well known polymeric biomaterials, such as polycaprolactone (PCL) and polylactic acid (PLA) as well as their nanocomposites. The preparation of

biomaterials in form of microparticles, 2D films and 3D porous scaffolds is presented in order to provide suitable bio-safe approaches for creating novel platforms with important applications in the biomedical field.

MATERIALS AND METHODS

Materials

PCL, polyethylene oxide (PEO) and gelatin were provided by Sigma-Alldrich (Madrid, Spain). PLA and a polylactic-co-caprolactone co-polymer (PLC) with 70/30 L-lactide/caprolactone were provided by Purac Biochem (Gorinchem, the Netherlands). EL (photoresist grade; purity ≥ 99.0%) was provided by Sigma-Aldrich (Madrid, Spain) and used without further purification. EA and ethanol were provided by Panreac (Barcelona, Spain). CO_2 with a purity grade of 99.995 wt% was purchased from Carburos Metálicos S.A. (Barcelona, Spain).

Methods

Different approaches have been used to process the biomaterials for the preparation of microparticles as well as 2D and 3D platforms.

Porous PCL microparticles were prepared in four steps, as described in [7]. First, PCL/EL solutions with a polymer concentration in the 5-30 wt% range were prepared at 70 °C maintaining magnetic stirring for 4 h. The solutions were then cooled down to a gelation temperature of either 6 or -15 ºC to induce the TIPS process. Gelled samples were further soaked in excess of water to extract the EL and to allow the PCL microparticles coagulation and setting at the bottom of the mould. The supernatant was then removed and the particles were washed three times with water to allow the complete removal of EL and the setting of the pore structure. Microparticles were finally dehydrated in ethanol and further dried at room temperature and ambient pressure. In order to exploit the possibility to further improve the control over the morphology and pore structure features of PCL microparticles, the TIPS process was also combined with a porogen leaching technique, selecting 10-20 μm gelatin particle as the solid porogen and gelatin-to-PCL concentrations ranging from 30 to 150 wt%.

2D platforms for cell culture study were obtained by solvent casting starting from polymeric solution in EL or EA, followed by drying in air or $scCO_2$. PCL, PEO and PLA were selected as main constituent for film fabrication and different processing conditions, namely polymer/solvent choice, polymer concentration and drying conditions were selected to modulate the final properties of the materials. The biomaterials were also blended to each other or with inorganic nano-particles in order to obtain hybrid multi-phase system with improved control over their morphological and topographical features.

Porous 3D scaffolds for cell culture and new tissue development were also prepared following two main approaches: gas foaming and phase separation. Foaming experiments were carried out on samples weighting 0.2 g and by using either pure $scCO_2$ or a $scCO_2$/EL mixture with an EL molar fraction of from 0 to 0.2 %. Blowing agent saturation was performed at different temperatures, in the range of 35 to 45 °C, and at pressures from 10 to 20 MPa. Samples were placed in a high-pressure autoclave (TharDesign, Pittsburgh, USA) with a total volume of 114 mL and provided of two sapphire windows that allowed for the visual on-time monitoring of the process. After saturation, foaming was induced by quenching the pressure to atmospheric. The pressure drop duration was controlled by using discharge capillaries of appropriate length and diameters, and ranged from to 0.5 to 10 min. For comparison, foaming experiments were

also carried out by pre-mixing the polymer with the solvents in order to form a paste and, subsequently foaming the system by using pure $scCO_2$.
Phase separation was also used to fabricate porous nanometre-scale fibrous PLA and PCL scaffolds. In Figure 1 it is reported a scheme of the process used for the preparation of PLA scaffolds along with representative SEM images of the samples.

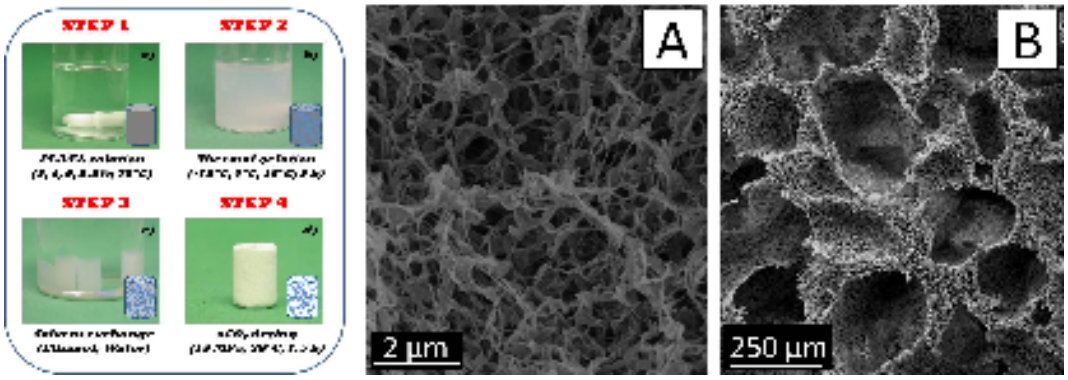

Figure 1. Scheme of the phase separation process used for the preparation of PLA nano-scale fibrous scaffolds. SEM micrographs of the scaffolds prepared by means of phase separation (A) with or (B) without using gelatin as particulate porogen [4].

In the step (1), PLA/EL solutions, with polymer concentration in the 3 - 5.5 wt% range, were prepared at 70 °C under magnetic stirring for 8 h. In the step (2), a definite amount of the starting solutions was added to aluminium moulds and transferred into a thermostatic bath (D8, Haake, Karlsruhe, Germany), pre-cooled to a temperature of either - 15, 0 or 15 ºC, for gelation during 3 h. In the step (3), gelled samples were removed from the mould and immersed into either distilled water or ethanol for solvent exchange. This step allows extracting the EL leading to polymer precipitation and scaffolds structure stabilization. The EL extraction was carried out by two different ways. In the first approach, the gels were soaked in excess of water and the medium changed 5 times up to the almost complete elimination of the EL. The obtained hydrogels were then soaked in ethanol to prepare PLA alcogels. In the second approach, the gels were directly immersed in ethanol to remove the EL. (4) In both cases, obtained alcogel samples were finally dried using $scCO_2$. Drying was carried out by using the high-pressure equipment described for foaming tests. Samples were placed inside of the autoclave on the top of a metallic support to allow for the addition of a magnetic stirrer at the bottom of the vessel. This set-up improved fluids mixing and reduced equilibrium time. After the reactor was charged with the gelled sample and sealed, the temperature was raised up to 39 ºC. Liquid CO_2 was subsequently pumped inside the vessel to raise the pressure up to 19 MPa, thus, ensuring the achievement of supercritical conditions. The operative temperature was selected to avoid crossing PLA glass transition temperature. These pressure/temperature conditions ensured the supercritical/gaseous state for the used scCO2/ethanol mixture, without passing through the liquid state. Samples were held at these conditions for 1.5 h. Finally, the vessel was depressurized, lasting the venting ca. 1 h.
In order to exploit the possibility of further improving the control of aerogels morphology and structure, the combination of TIPS with porogen leaching (PL) techniques was assessed. In this approach, in the step (1) the polymeric solution was mixed with gelatin particles in a 1.2 v/wt% or allowed to penetrate inside a porous gelatin template previously prepared. The obtained samples were maintained at 0 ºC for 3 h for gelation and, subsequently, the EL was extracted by soaking in ethanol. The obtained material was further soaked in water at 40 ºC

for 2 days to selectively extract the gelatin particles. The final PLA scaffolds were obtained by exchanging the water with ethanol and drying the alcogel following the previously described protocol in step (4).

Characterization

Thermo-gravimetric (TGA-DTGA) analysis was performed for materials before and after processing to evaluate the effect of the developed process on the thermal stability of the polymer. The morphology and crystalline structure of the samples were assessed by means of polarized microscopy and scanning electron microscope (SEM) and transmission electron microscope (TEM) analyses. The porosity of the scaffolds was determined by geometrical calculation from the mass and volume measurements. The mean pore size and pore size distribution of the scaffolds were evaluated by Image (Image J®) analysis. Nano-metric size pore structure characteristics of the samples were studied by low temperature N_2 adsorption-desorption analysis. *In vitro* biological characterizations were also carried out on selected biomaterials to assess their biocompatibility for tissue engineering applications.

RESULTS

In this work we reported the novel design and fabrication of different type of biomaterials by means of bio-based and clean approaches.

A series of porous nanometre-scale fibrous PLA scaffolds was fabricated by varying the composition of the polymeric solution, the gelation temperature and the extraction medium. SEM images reported in Figures 1A and B show the morphology of porous PLA scaffolds prepared by using phase separation and phase separation combined with porogen leaching, respectively. As shown, the scaffolds were characterized by a fibrous structure, with fibre diameters in the submicron range, and porosity higher than 90% (Figure 1A). The possibility to design and manufacture porous PLA scaffolds characterized by multi-scaled pore structures is of great importance in tissue engineering, were scaffolds with pores larger than 100 μm are necessaries to allow for the three dimensional adhesion and colonization with cells, as well as for new tissue development *in vitro* and *in vivo*. The addition of the micrometric particulate porogen effectively allowed for the fabrication of multi-scaled porous PLA scaffolds with large pores, in the order of 300 μm, replicating the size of the starting gelatin particles. Furthermore, by optimizing the phase separation process, it was possible to induce high pore interconnectivity as well as to recreate a nanometre-scale fibrous architecture on the pore walls (Figure 1B).

The second process which was investigated is gas foaming by using $scCO_2$ and mixtures of $scCO_2$ and a co-solvent as blowing agent. In Figure 2 are reported SEM images showing the morphology of porous PCL, PLA and PLC scaffolds prepared by using different blowing agent mixture compositions. As shown, by comparing the SEM images of Figures 2A and D, it could be observed that the addition of EL to the blowing agent mixture was essential to improve PCL foaming. Furthermore, scaffold morphology was characterized by the presence of pores of approximately 30 μm alongside with a nano-fibrous structure on the entire pore walls (Figure 2D). For both PLA and PLC polymeric materials, the processing conditions selected resulted in scaffolds with a rather homogeneous morphology, while the addition of EL to the blowing agent mixture clearly enhanced the size of the pores of the foams (Figures 2B, C, E and F).

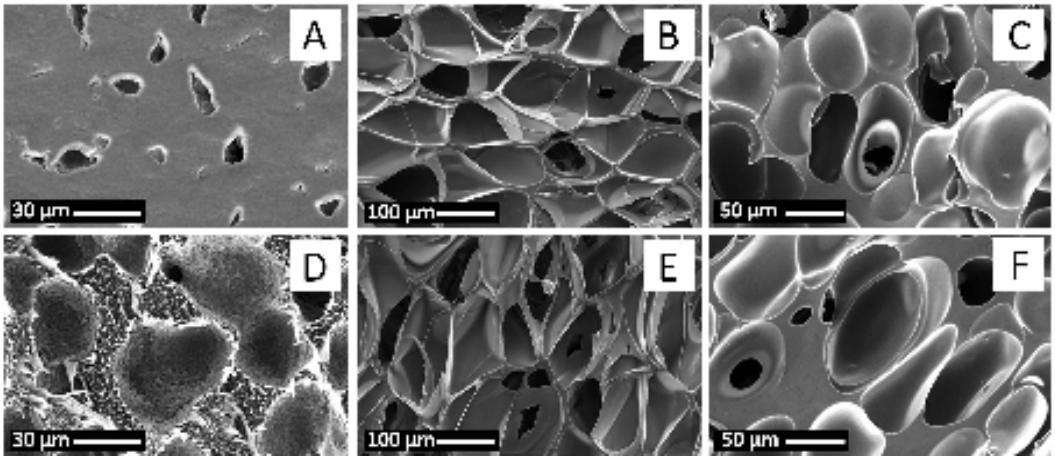

Figure 2. SEM images of porous scaffolds prepared by gas foaming process and by using (A, B and C) $scCO_2$ and (D, E and F) $scCO_2$/EL as blowing agent mixtures. (A and B) PCL, (C and D) PLA and (E and F) PLC scaffolds [10].

CONCLUSION

In this work, bio-based and sustainable approaches to manufacture novel biomedical devices, namely porous microparticles as well as 2D and 3D platforms for tissue engineering were reported. In particular, porous biomaterials were fabricated by means of thermal induced phase separation or gas foaming processes and by using mainly $scCO_2$ combined with EL and EA as clean porogen agents. By controlling the operating conditions during biomaterials process we were able to design novel biomaterials with controlled features down to the nanometric size scale and, finally, obtain biomimetic platforms for cell culture.

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