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Subcritical carbon dioxide foaming of polycaprolactone for bone tissue regeneration

Rui M. Duarte [a,b,d,e], Jorge Correia-Pinto [a,b,d,f], Rui L. Reis [c,d], Ana Rita C. Duarte [c,d*1]

[a] School of Medicine, University of Minho, Campus de Gualtar 4710-057, Braga, Portugal

[b] Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar 4710-057 Braga, Portugal

[c] 3B´s Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Avepark – Parque de Ciência e Tecnologia, 4805-017 Barco GMR, Portugal

[d] ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal

[e] Orthopedic Surgery Department, Hospital de Braga, Sete Fontes-São Victor, 4710-243 Braga, Portugal

[f] Pediatric Surgery Department, Hospital de Braga, Sete Fontes-São Victor, 4710-243 Braga, Portugal

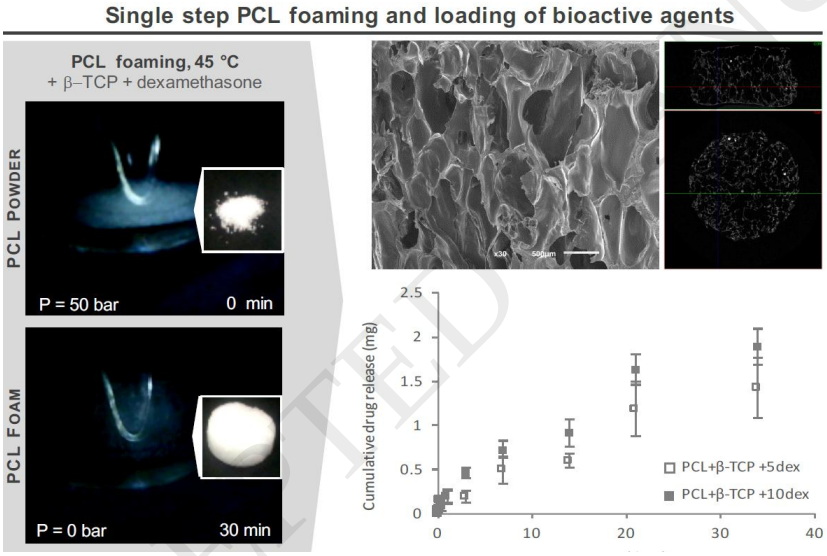
*** Corresponding Author:**

Ana Rita C. Duarte, aduarte@dep.uminho.pt

[1]Present Address: Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal. aduarte@fct.unl.pt

3B´s Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Avepark – Parque de Ciência e Tecnologia, 4805-017 Barco GMR, Portugal

Graphical Abstract



Highlights-

- A physiologically friendly polycaprolactone foaming procedure
- Use of dense CO_2 as foaming agent for creation of porous three-dimensional structures of polycaprolactone without supercritical conditions
- A one step approach for foaming and loading of polycaprolactone with bioactive agents such as ß-tricalcium phosphate and dexamethasone

Abstract

The preparation of three-dimensional polycaprolactone scaffolds using dense CO_2 as foaming agent, without supercritical conditions, was evaluated in this study towards future applications in bone repair. Herein, 3D foams were obtained at 5.0 MPa and 45 °C. To induce bioactivity, β-tricalcium phosphate (β-TCP, 10 wt%) and dexamethasone (5 and 10 wt%) were dispersed in the scaffolds. Foams revealed a pore size range of 164-882 μm, 73-99% porosity and 79-99% interconnectivity, assessed by micro-computed tomography, and a Young modulus of 1.76-2.92 MPa. Dexamethasone did not impair morphology of the matrices in comparison with PCL+β-TCP, which presented a water uptake of nearly 100% after 14 days. A sustained release of dexamethasone was achieved over 35 days in physiologic solution. This study reports the feasibility of using dense CO_2 to produce in one-step a porous matrix loaded with active agents opening new possibilities towards injectable systems for *in situ* foaming.

Keywords

Polycaprolactone; carbon dioxide; subcritical; bone regeneration; scaffolds; bioactive agents

1. Introduction

The development of 3D architectures for tissue engineering has challenged many researchers involved in different fields of knowledge. The ability to create a three-dimensional scaffold, which meets the morphological, mechanical, chemical and biological requirements for good cellular performance and tissue integration, is not straightforward. In this sense, the pursue for different processing techniques which can offer the most appropriate construct for the application of such purpose has been the goal of many different works reported in the literature. One of the techniques that has been extensively studied for polymer processing is the use of gas and/or supercritical foaming. This technique was firstly proposed by Mooney and co-workers back in 1996 [1] to produce a highly porous and interconnected structure for tissue engineering and regenerative medicine. In the work reported, foaming of poly(-lactic-co-glycolic acid) without the use of organic solvents was described. The thermodynamic principle underlying the gas foaming is the plasticization effect of the gas on the polymeric matrix, decreasing its glass transition and/or melting temperature. Upon decompression cells start to nucleate and the pores are formed [2–6]. Carbon dioxide is the supercritical fluid used per excellence, particularly due to its low critical properties (P_c= 7.4 MPa and T_c= 31 °C), but also due to its innocuity. The main constraint of the gas/supercritical foaming technique is, however, the limited ability of carbon dioxide to decrease the glass transition temperature of crystalline polymers, hence this processing can only be applied to amorphous or semi-crystalline polymers and the extent of foaming will be directly related to the affinity of carbon dioxide to the polymer.

Since the pioneering work of Mooney, different polymers were tested. Particularly interesting for biomedical applications are different derivatives of polyesters such as poly(L-lactic acid) [7,8], poly(DL-lactic acid) [9,10], poly(lactic-co-glycolic acid) with different ratios of lactic and glycolic [9,11] and polycaprolactone (PCL). In this work we selected polycaprolactone, a FDA-approved polymer used for several biomedical applications, which has attracted attention in bone tissue engineering for its biocompatibility, bioabsorbability and mechanical properties

[12]. Particularly in spinal fusion, PCL has demonstrated to yield successful bone fusion and maturation of newly formed bone, in large animal models such as pig [13,14] and sheep [15,16], outperforming bone graft [17,18].

Several techniques are used to process PCL and PCL composites towards achieving optimal performing physicochemical properties for bone applications, such techniques including particulate leaching [19], compressive moulding [20], melt spinning [21], wet spinning [22], electrospinning [23], or fused deposition modeling (FDM) [13] / 3D-printing [24] , which yield a pre-fabricated scaffold suitable for implantation. The use of gas and/or supercritical foaming technique and experimental conditions proposed herein, brings the possibility of foam injection, for in situ formation of the 3D porous scaffold. The settled material shall achieve the specific format and dimensions of the osseous defect. Multiple manuscripts have reported different experimental conditions for gas and/or supercritical foaming of PCL, including polymers of several molecular weights and the presence of additives, as detailed in Table 1.

Most of the work refers to supercritical fluid foaming, i.e. conditions in which carbon dioxide was used above its critical parameters of pressure and temperature. There are however two exceptions. Cotugno *et al* [26] report the foaming of polycaprolactone, in a two-step approach. The polymer is initially molten at temperatures higher than its melting temperature and pressurized with CO_2. Afterwards the system is cooled and, upon depressurization of the system to ambient pressure, the foaming takes place. In this work [26] it is however difficult to assess which experiments are carried out at sub or supercritical conditions as the final pressure, before depressurization is not mentioned. Another work reported in the literature, by Di Maio and co-workers [40] presents several experiments were carried out to foam polycaprolactone in a temperature range from 28-45 °C and a pressure range from 2.8-5.5 MPa. Although the manuscript presented does not provide extensive morphological characterization of the foams generated, it is possible to conclude that the foams prepared have small pore size diameter in

the range of 20-80 μm and a density between 0.05 and 0.25 g/cm^3. The different approaches followed by Di Maio *et al* [40] aims the understanding of the effect of the operating conditions on the morphology of the scaffolds, but also the thermodynamics underlying the process. A more applied research has been reported by Annabi [37] and De Matos [41] which relates specifically to the preparation PCL based 3D architectures for tissue engineering and regenerative medicine.

In 2003 David Tomasko and co-workers [43] have highlighted the main constrains of using carbon dioxide as foaming agent claiming that "The challenges of CO_2 as a foaming agent are associated with the higher pressure operation, dimensional instability during the foam-shaping process (…)" [43]. 15 years later, the industry is still reluctant when it comes to high-pressure and supercritical technology. Despite the significant advances at the scale-up and translation from lab to bench scale the perception that working under high pressures and the requirement of sophisticated and expensive equipment, continues to hinder the use of supercritical fluid technologies at a larger scale.

In this work, we evaluated the possibility to prepare 3D scaffolds from polycaprolactone under subcritical carbon dioxide atmosphere. The possibility to use milder processing conditions may open new strategies in bone regeneration, particularly through the development of a portable surgical tool for *in situ* foaming and foam delivery.

2. Materials and methods

2.1. Materials

Poly-ε-caprolactone (PCL) (Mn 45000 Da and 80000 Da) in granular form was obtained from Sigma Aldrich and milled to powder using an ultra-centrifugal mill (Retsch ZM200) under liquid nitrogen. β-tricalcium phosphate (β-TCP) (CAS 7758-87-4) and dexamethasone (dex) (CAS50-02-

2) were also obtained from Sigma Aldrich. Carbon dioxide (99.998 mol %) was supplied by Air liquid. All reagents were used as received without further purification.

2.2. Subcritical carbon dioxide foaming

The scaffolds were prepared by subcritical carbon dioxide foaming at 5.0 MPa and 45 °C. In each experiment c.a. 500 mg of PCL was loaded in a stainless steel cylindrical mold with 2 cm inner diameter and 1 cm height, which was placed inside the high-pressure vessel (2.5 cm inner diameter, 40 cm height) (Figure 1). The vessel was heated by means of an electric thin band heater (OGDEN) connected to a temperature controller, which maintained the temperature within ± 1 °C. The system was pressurized with carbon dioxide until the operational pressure was attained inside the vessel, measured with a pressure transducer. The system was closed for two hours to allow the plasticization of the polymer. Afterwards the system was slowly depressurized (depressurization rate ~0.15 MPa/min).

When β-TCP and dexamethasone were loaded in the 3D construct, they were previously physically mixed with the PCL polymer and afterwards loaded in the mold. The foaming procedure was the same as described above.

Figure 1.

A summary of the samples prepared is listed in Table 2:

2.3. Characterization of the 3D structures

2.3.1. Scanning electron microscopy (SEM)

Porous matrices obtained from the different formulations were observed by a Leica Cambridge S360 Scanning Electron Microscope (SEM). Cross sections of the specimens were examined after fracturing in liquid nitrogen. The matrices were fixed by mutual conductive adhesive tape on aluminum stubs and covered with gold palladium using a sputter coater.

2.3.2. Micro-computed tomography (micro-CT)

The morphological structure and the calculation of the morphometric parameters that characterize the samples were evaluated by micro-computed tomography (micro-CT) using a Skyscan 1272 equipment (Bruker, Germany) with penetrative X-rays of 50 KeV and 200 µA, in high-resolution mode with a pixel size of 21.6 µm. A CT analyzer (v1.15.4.0, 2012-2015, Bruker Micro-CT) was used to calculate the parameters from the 2D images of the matrices.

2.3.3. Compressive mechanical analysis

Compressive mechanical analysis of the materials produced were measured using an INSTRON 5540 (Instron Int. Ltd, High Wycombe, UK) universal testing machine with a load cell of 1 kN. The scaffolds were cut in cylindrical shape with 5 mm diameter and 4 mm height. Compression testing was carried out at a crosshead speed of 2 $mm.min^{-1}$, until a maximum reduction in sample weight of 60 %. The compressive modulus is defined as the initial linear modulus on the stress/strain curves. The data presented is the result of the average of at least five measurements.

2.4. Water uptake and degradation studies

The water uptake ability of the matrices prepared was assessed for a period up to 14 days. Scaffolds of PCL and PCL + β-TCP prepared by dense gas foaming were weighed and immersed in 5 mL of an isotonic solution (Phosphate Buffered Saline, PBS) at pH = 7.4. The samples were placed in a water bath at 37 °C. After predetermined periods of time (1, 3, 7 and 14 days) the samples were weighed in order to determine the water uptake of the scaffolds.

Water uptake was determined using the following equation (1):

$$\%\ \text{water uptake} = \frac{w_w - w_i}{w_i} \times 100 \quad (1)$$

where w_w is the weight of the wet sample and w_i is the weight of the initial sample.

The pH of each solution was also measured in order to determine the effect of polymer degradation on the acidity of the medium.

After immersion for 28 days the samples were dried at room temperature and weighed to determine the weight loss, which was calculated according to equation (2):

$$\% \text{ weight loss} = \left| \frac{w_f - w_i}{w_i} \right| \times 100 \quad (2)$$

where w_f is the final weight of the dry sample after immersion and w_i is the initial weight of the sample.

2.5. Drug release studies

In vitro release studies of dexamethasone from PCL foams were performed by incubating the drug-loaded matrices in 20 mL of PBS, at pH 7.4 under horizontal shaking at 37 °C [23,44]. The release was performed in sink conditions. At appropriate time intervals (5 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 24 h, 72 h, 7 days, 14 days, 21 days, 34 days), aliquots of 300 μL were withdrawn, and fresh volume of PBS was added to the suspension to replace the sample. The samples were quantified by UV-spectroscopy on a multi-well microplate reader (Synergy HT, Bio-Tek Instruments), using a quartz 96 well-plate. The optical density of dexamethasone was read at 245 nm. The results were presented as an average of three measurements.

2.6. Cytotoxicity studies

An immortalized mouse lung fibroblasts cell line (L929), from European Collection of Cell Cultures, UK, was maintained in basal culture medium DMEM (Dulbecco's modified Eagle's medium; Sigma–Aldrich, Germany), supplemented with 10% FBS (heat-inactivated fetal bovine serum, Biochrom AG, Germany) and 1% A/B (antibiotic–antimycotic solution, Gibco, UK). Confluent L929 cells were harvested, seeded in a 96 well plate at 1 x 10^3 cells/well using supplemented DMEM medium, and cultured for 24h. The indirect contact was performed by replacing the culture medium with leachables of the materials. The leachables were obtained after 24 hours of extraction using a ratio 100 mg of material in 1 mL supplemented DMEM. The samples were cultured for 48 hours under a 5% CO_2 atmosphere at 37 °C, after which cell

metabolic activity was determined by the MTS assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA). Absorbance was measured at 490 nm using a microplate reader (Synergie HT, Bio-Tek, USA). Optical density was determined for sample and compared to polystyrene tissue culture plate, used as a negative control and latex extract used as a positive control. All cytotoxicity screening tests were performed using three replicates.

3. Results and Discussion

In this work, we have evaluated the possibility to foam polycaprolactone under subcritical carbon dioxide atmosphere. Literature review (Table 1) suggests that successful foaming of this polymer requires conditions above the critical parameters of carbon dioxide and little has been reported in what concerns the use of carbon dioxide at lower P, T conditions. The first variable optimized was the molecular weight of polycaprolactone as gas foaming is highly dependent on the molecular weight of the polymers. In this work, we tested different polycaprolactone polymers, with molecular weights of 45 000 and 80 000 Da. The experiments carried out have shown that under the processing conditions aimed in this study, i.e., below 5.0 MPa and 50 °C, polycaprolactone with a molecular weight of 80 000 Da did not foam. According to literature (Table 1), much higher conditions are required to achieve foaming of PCL 80 000 Da, i.e: pressure up to 20.0 MPa and/or temperatures above the melting temperature of the polymer. On the other hand, PCL 45 000 Da processed under 5.0 MPa and 50 °C could be foamed and was, for this reason, selected to proceed the optimization process. Two different pressures (4.0 and 5.0 MPa) and different temperatures (between 37 to 50 °C) were tested, in order to further optimize the gas foaming process and determine the best operating conditions to prepare a 3D architecture able to meet the requirements set for bone tissue engineering. These experiments were performed with an exposure time of 2 hours. Figure 2 highlights the different set of

operating conditions reported in the literature for the foaming of PCL and the ones tested in this work.

Figure 2.

At 4.0 MPa and 45 °C, sintering of the particles takes place. Sintering under dense carbon dioxide atmosphere has been reported in the literature and relies on a minor plasticization effect of carbon dioxide [45]. In the experiments performed the polymer particles are fused, but a very fragile structure is obtained. The structure cannot be handled without compromising its integrity. On the other hand, increasing pressure to 5.0 MPa and 37 °C, a homogeneous and robust architecture is obtained, however we can consider that under these conditions still sintering and not foaming occurred. It is only when temperature is increased to 45 °C, with a carbon dioxide pressure of 5.0 MPa that foaming takes place. From the same amount of starting material, the sample prepared at 5.0 MPa and 45 °C has expanded much more than the sample prepared at 37 °C, presenting a larger volume and hence, lower density. From these results, we concluded that the best operating conditions were 45 °C and 5.0 MPa and these were therefore used for further processing of the constructs.

The optimization of the required contact time for plasticization is another parameter which is crucial for the success of the polymer foaming. The plasticization of polycaprolactone was assessed using a high-pressure visual cell with a sapphire window. Imaging of the process was performed through sequential images which were captured in time lapse mode by computer software controlled program equipped with a Logitech (HD1080P) camera. Figure 3 shows selected images of the foaming process as a function of time.

Figure 3.

The images demonstrate that upon pressurization of the high-pressure cell, the polymer starts to plasticize after 10 minutes and after 30 minutes it is completely in the molten state. In the

decompression cycle, it is observed that the foaming occurs in the late stage of decompression of the vessel, when pressure is below 1.0 MPa. These observations are particularly relevant to optimize processing time. Fanovich and co-workers reported visual observations of the gas foaming for the system with polycaprolactone [39] and evaluated the temperature effect at 35 and 40 °C and pressure of 15.0 up to 20.0 MPa. Under these conditions the PCL scaffolds are molten after 30 minutes in contact with the supercritical fluid. No major differences are observed in terms of the time requested for polymer plasticization/melting either in sub or supercritical conditions. This is particularly true when large differences in CO_2 density are compared. For instance, in our work, the CO_2 density of the gas phase is ~0.104 g/cm^3 (determined using a web computation tool provided by Penn State – Earth and Mineral Sciences Energy Institute), while in the work by Fanovich, CO_2 density used was 0.839 g/cm^3 in the experiments carried out in supercritical conditions (35 °C and 15.0 MPa).

The morphological analysis of the 3D constructs produced was evaluated by scanning electron microscopy and by micro computed tomography. Figure 4 presents the images from the cross sections observed.

Figure 4.

The scaffolds present a very homogenous architecture with uniform pore distribution in all formulations, as observed by SEM. Differences in pore size and interconnectivity among formulations is evident by SEM images and further confirmed by micro-CT characterization (Table 3). The values of the different morphological parameters were determined by image analysis after micro-computed tomography. The interconnectivity of the scaffold is calculated according to the formula: $I = \left[\left(V_{totalpore} - V_{disconnectedpore}\right) / V_{totalpore}\right] \times 100$, where the volume of the disconnected pore stands for the disconnected pore volume which was defined to be higher than 50 μm. The degree of isotropy is a measure of the 3D symmetry or the presence or absence

of preferential alignment of structures along a particular directional axis. Bone is known to be an anisotropic structure and this morphological characteristic of trabecular bone also plays an important role in the mechanical strength of the architecture produced [46].

In terms of porosity, the values obtained in this work are in accordance with what is reported in the literature (table 1), which is between 60 and 90%. In some cases, the porosity of the matrices produced is even slightly higher than what has been previously reported. There is one exception, presented by de Matos, that reports porosity values between 20 and 50%. However, the porosity reported was determined by mercury intrusion porosimetry and this may explain the low porosity reported by the authors [41]. Pore size is the parameter which can be compared from a larger set of data. Figure 5 presents a schematic representation of the pore size distribution of the matrices reported in the literature in comparison to the ones obtained in this work. The size of the bubbles represents the range of pore size obtained at the different conditions employed in each cited work. As it can be observed the pore size is much higher when subcritical P, T conditions are used in the foaming process (dark grey data set), possibly due to a distinct expansion profile of the foam.

Figure 5.

Most important however, is the establishment of a comparison with the data from trabecular bone reported in the literature [47]. The morphological analysis of the scaffolds is in very good agreement with this data (table 3) suggesting that the scaffolds prepared under subcritical carbon dioxide atmosphere can be interesting for bone regeneration applications.

The 3D reconstruction of the structures prepared can also be obtained by micro-computed tomography. Figure 6 presents the reconstructed 3D architectures of the scaffolds PCL and PCL

+ β–TCP. This technique also allows the visualization of the distribution of β–TCP within the construct due to the inherently different densities of the polymer and the inorganic/ ceramic part. As it can be observed the foaming with β–TCP particles dispersed renders a uniform dispersion of the ceramic part within the structure.

Figure 6.

The mechanical properties of the 3D constructs produced were evaluated in compression mode. For an open cellular material, usually stress-strain curves are characterized by three regions, a linear elastic regime, a plateau and a densification region [48]. Figure 7 displays a representative image of the deformation that occurs upon loading and a scheme of the typical stress-strain deformation of a porous polymeric structure ("Example"). The elastic regime is characterized as a reversible region where the deformation applied can be reversed, the cell walls bend, but have the ability to fully recover the shape when the load or stress applied is removed. The plateau characteristic of the initial plastic region is a region characterized by the fracture of some cells and the disruption of the structure. If the force continues to be applied a densification region is observed, cell walls collapse and the deformation is irreversible [49].

A closer look at the initial mechanical properties of the two structures (Figure 7) highlights the fact that both PCL and PCL + β -TCP structures prepared under these operating conditions have a limited elastic and plateau region, and the samples present a plastic behavior typical of elastomeric materials. The elastic regime of both PCL and PCL + β-TCP is limited to up to 1 and 5% strain, respectively. It is hence, in this region that the elastic modulus is determined. The Young modulus calculated from the initial slope of the curve (up to 1% strain) corresponds to 2.97 ± 0.7 MPa and 1.76 ± 0.7 MPa for PCL and PCL + β–TCP, respectively.

Figure 7.

De Matos and co-workers report the compressive modulus of composite PCL + mesoporous silica particles scaffolds prepared by supercritical fluid foaming at 35 °C and 14.0 to 25.0 MPa. In their work the reported scaffolds present a compressive modulus of 20-150 MPa [45]. It would perhaps be expected that β–TCP could enhance the mechanical properties of the scaffold, however, the mechanical properties are also strictly connected to the morphological features of the three-dimensional architectures. In our work, the mechanical properties of the scaffolds produced are much lower than those reported by De Matos, but the morphological features are also different, the porosity of the PCL + β–TCP structures herein prepared have a much higher porosity value (90 vs 20-50%) and the content of ceramic filler much lower (10 vs 30 wt%). Hence, the higher porosity, pore size and interconnectivity observed for the PCL + β–TCP may explain this decrease in the Young modulus. Other authors have reported the decrease in mechanical properties after the addition of hydroxyapatite or bioglass in PCL scaffolds [50–52]. The production of hybrid scaffolds loaded with inorganic fillers has not only the purpose to reinforce the structure but also, and most importantly to create a bioactive structure. The desired scaffold shall result from a fine balance between mechanical properties and bioactivity. Mechanical properties of bone, and particularly those in spine surgery, far exceeds the values herein obtained. Bone has very high mechanical properties which depend greatly on its hierarchical structure. While cortical bone is a compact structure, cancellous bone is composed of irregular, sinuous convolutions of lamellae, and these differences are highly noticeable in the elastic modulus of 0.1 to 0.5 GPa for cancellous bone and 12 to 18 GPa for cortical bone; and a compressive strength situated between 130 and 180 MPa for cortical bone [53]. Chen and co-workers [52] report the development of porous foams as bone augments and grafts, and even though the mechanical properties of the polycaprolactone scaffolds produced are relatively low when compared to cancellous one, the *in vivo* study performed demonstrated a good performance of the scaffolds [52]. It should be highlighted that it is in fact, not only one property but a combination of different features that may offer distinct advantages over other materials,

particularly in which concerns the enhancement of bone regeneration. Polymeric scaffolds designed for tissue engineering and regenerative medicine applications have to meet a particular set of requirements, highly tailored towards compatibility and integration with surrounding tissue at implantation site. On what regards morphological properties, scaffolds for bone repair should present open and interconnected pores and adequate pore size, allowing for cell growth and vascularization. Scaffolds should ensure sufficient mechanical strength to withstand the tissue-specific biological forces and maintain cell physical integrity. Adequate surface properties, both chemically and topographically, should promote cell adhesion and proliferation. When working with biodegradable structures, its degradation rate should match the growth rate of the neotissue. Sterilization of the final product should be thought early in scaffold design, so that it will not compromise the structure properties [54–56]. It is, hence, extremely difficult to produce an implant that meets all the ideal criteria. Furthermore, as the characteristics and features of the matrices are related, sometimes the enhancement of one property may compromise another. For all the above, the aim is to develop scaffolds which, despite being far from the optimal conditions, show evidences that may lead to motivating outputs when implanted *in vivo*.

Another important feature, related with the surface properties of the scaffolds prepared is the water uptake ability. The measurement of the water uptake ability provides an indication of the bulk hydrophilicity and thus the susceptibility of the scaffolds to suffer hydrolysis. Furthermore, this feature is also related with the diffusion of nutrients and oxygen to the cells together with the elimination of cell wastes, which occurs in an aqueous environment. Figure 8 (A) presents the water uptake of the PCL and PCL + β-TCP formulations tested. Both formulations present different water uptake abilities, where PCL + β-TCP attains nearly 70% at the first 24 h, reaching 100% by the end of the 2-week time-frame, while PCL foams achieve nearly half water uptake at same time-points. After 14 days of immersion, the water uptake has reached the plateau region for PCL (at approx. 50%), yet not for PCL + β-TCP, which shall be able to uptake more

water at longer time-points. These observations can be justified by the differences observed in the morphological properties of the scaffolds. Larger pores and higher porosity and interconnectivity facilitate water diffusion into the bulk of the sample contributing to an enhancement of the water uptake ability of the scaffold. Additionally, the presence of β-TCP, has been reported to promote higher hydrophilicity of polycaprolactone scaffolds, as described by Zhou and co-workers [57]. One of the concerns when using synthetic polyesters is the possibility of degradation by hydrolysis and production of acidic residues due to the cleavage of the carboxylic groups [21]. For this reason, it is important to follow the pH of the solution in which the scaffolds are immersed. In this work, we have evaluated the changes in pH of a physiological solution throughout 28 days. As it can be seen in figure 8 (B), the pH of the solution did not change significantly upon the duration of this study. Furthermore, we have quantified the degree of degradation by weight loss and after 28 days, the materials present an average degradation of 1 wt. %, for all samples tested. These results demonstrate the high stability of polycaprolactone scaffolds in physiological solution. It has been recognized that polycaprolactone presents a long degradation time, which in vivo may vary between two and four years, given that its degradation occurs mostly by hydrolytic processes [7,48].

Figure 8.

It has long been recognized that scaffolds for tissue engineering should move from a merely inert structure towards a bioactive support, which is able to promote the necessary cues for an improved regeneration process. In addition to a ceramic material, we have evaluated the possibility to homogeneously disperse a second bioactive agent, namely dexamethasone. Dexamethasone was chosen due to the potential of this drug to direct stem cell differentiation towards the osteogenic lineage [58,59]. Two different drug concentrations were evaluated, and as demonstrated by the morphological analysis (Table 2), the presence of both β-TCP and dexamethasone did not compromise the porosity and interconnectivity of PCL scaffolds

produced under dense carbon dioxide atmosphere. In fact, a more porous and interconnected structure with larger pores seems to be obtained with this supplementation, which could be the result of distinct expansion profile of the foam. This cause-effect shall be further analyzed in future studies. The morphological differences of each formulation are expected to have an impact on mechanical performance upon application, whereas a fine balance between supplementation of bioactive agents and such mechanical properties shall be achieved. It was found that, for instance addition of β-TCP did not result in augmented young modulus upon compression as compared to non-supplemented PCL, possibly due to the higher porosity and pore size. Nevertheless, its morphology and bioactivity shall offer a more osteoinductive and osteoconductive environment aimed towards improved osteointegration. Pore size and porosity are intimately related with surface area, whereas structures with higher surface area, are more exposed to water molecules which shall lead to faster hydrolytic degradation of the PCL component. These can be fine-tuned in order to reach the best performing structure on what regards matching new bone formation and scaffold degradation rates, as well as controlled release of bioactive agents such as the dexamethasone. The *in vitro* drug release profile was followed up to 35 days in an isotonic physiological solution and the results are presented in figure 9.

Figure 9.

Dexamethasone was sustainably released from the scaffolds within one month. Up to 24 hours there are no relevant differences between the two systems, loaded with 5 or 10 wt% of dexamethasone. The release profile presented a lag time of 2 hours before dexamethasone could be detected in the release media. Between 2 and 6 hours there is an eventual burst release as the drug concentration in solution increased. From this point onwards, there is a controlled release of the drug from the scaffold. The release profile of both structures presents a parallel trend, suggesting that the mechanisms underlying the release are the same and the differences

encountered are only due to the differences in the loaded concentrations. At the end of this study, and as it would be expected, the cumulative amount of dexamethasone in solution is higher for the samples prepared with 10 wt% dex, however, these samples have a slower release rate than the scaffolds impregnated with 5 wt% dex. After 35 days, the scaffolds loaded with 5 wt% have released 100% of the drug impregnated while the scaffolds loaded with 10 wt% have released approximately 75% of the drug.

One of the most important requirements in a scaffold for tissue engineering and regenerative medicine is the biocompatibility of the material. Hence, the cytotoxicity of the materials prepared was evaluated by an indirect contact in which the cells are cultured in the presence of the materials leachables extracted for 24 hours. Cell viability determined by MTS assay was determined as a function of the cell viability of the cells cultured with DMEM culture media. The results for the different formulations are presented in figure 10.

Figure 10.

The results demonstrate that the leachables of the different structures prepared do not compromise the viability and metabolic activity of the cells and hence could be used as scaffolds for tissue engineering applications.

4. Conclusions

In this work, we evaluated the possibility to prepare 3D scaffolds from polycaprolactone under dense subcritical carbon dioxide atmosphere. Processing technologies, carried out to pressures up to 5.0 MPa can encourage the development of new materials, the use of cheaper equipment, eliminating the need for cooling systems, liquid pumps, compressors and all the negative connotation of the “high-pressure” concept without the need to go up to supercritical conditions. Unlike the studies reported in the literature, it was possible to create porous

structures without the need to use supercritical condition, i.e pressures below 74 MPa. The structures prepared present interesting morphological and mechanical properties to be used as scaffolds in bone tissue engineering and regenerative medicine and open new perspectives for the treatment of bone defects by *in situ* foaming, through the development of a portable surgical tool. Furthermore, osteoconductive and osteoinductive active agents were incorporated into PCL in a single operating step: β-tricalcium phosphate and dexamethasone were homogeneously dispersed within the PCL matrix. Dexamethasone was released sustainably from the constructs, with a higher delivery rate during the first week of the study, enhancing the functionality of the PCL scaffold. The cytotoxicity of the materials produced was also studied and the different formulations tested did not show any compromise of the cell viability after 48 hours of contact. Overall, the results obtained prove that this novel hybrid structure might be a promising approach as a multifunctional template for regenerative medicine applications.

Declarations of interest: none.

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References

[1] D.J. Mooney, D.F. Baldwin, N.P. Suh, J.P. Vacanti, R. Langer, Novel approach to fabricate porous sponges of poly(D,L-lactic-co-glycolic acid) without the use of organic solvents, Biomaterials. 17 (1996) 1417–1422. doi:10.1016/0142-9612(96)87284-X.

[2] A.R.C. Duarte, J.F. Mano, R.L. Reis, Perspectives on: Supercritical Fluid Technology for 3D Tissue Engineering Scaffold Applications, J. Bioact. Compat. Polym. . 24 (2009) 385–400. doi:10.1177/0883911509105796.

[3] L.J.M. Jacobs, M.F. Kemmere, J.T.F. Keurentjes, Sustainable polymer foaming using high pressure carbon dioxide: a review on fundamentals, processes and applications, Green Chem. 10 (2008) 731. doi:10.1039/b801895b.

[4] S.P. Nalawade, F. Picchioni, L.P.B.M. Janssen, Supercritical carbon dioxide as a green solvent for processing polymer melts: Processing aspects and applications, Prog. Polym. Sci. 31 (2006) 19–43. doi:10.1016/j.progpolymsci.2005.08.002.

[5] S.K. Goel, E.J. Beckman, Generation of microcellular polymeric foams using supercritical carbon dioxide. I: Effect of pressure and temperature on nucleation, Polym. Eng. Sci. 34 (1994) 1137–1147. doi:10.1002/pen.760341407.

[6] A.I. Cooper, Polymer synthesis and processing using supercritical carbon dioxide, J. Mater. Chem. 10 (2000) 207–234. doi:10.1039/a906486i.

[7] L.M. Mathieu, M.O. Montjovent, P.E. Bourban, D.P. Pioletti, J.A.E. Månson, Bioresorbable composites prepared by supercritical fluid foaming, J. Biomed. Mater. Res. - Part A. 75 (2005) 89–97. doi:10.1002/jbm.a.30385.

[8] G. Georgiou, L. Mathieu, D.P. Pioletti, P.E. Bourban, J.A.E. Månson, J.C. Knowles, S.N. Nazhat, Polylactic acid-phosphate glass composite foams as scaffolds for bone tissue

engineering, J. Biomed. Mater. Res. - Part B Appl. Biomater. 80 (2007) 322–331. doi:10.1002/jbm.b.30600.

[9] H. Tai, M.L. Mather, D. Howard, W. Wang, L.J. White, J.A. Crowe, S.P. Morgan, A. Chandra, D.J. Williams, S.M. Howdle, K.M. Shakesheff, Control of pore size and structure of tissue engineering scaffolds produced by supercritical fluid processing, Eur. Cells Mater. 14 (2007) 64–76. doi:vol014a07 [pii].

[10] V.E. Santo, A.R.C. Duarte, E.G. Popa, M.E. Gomes, J.F. Mano, R.L. Reis, Enhancement of osteogenic differentiation of human adipose derived stem cells by the controlled release of platelet lysates from hybrid scaffolds produced by supercritical fluid foaming, J. Control. Release. 162 (2012) 19–27. doi:10.1016/j.jconrel.2012.06.001.

[11] K.C. Baker, R. Bellair, M. Manitiu, H.N. Herkowitz, R.M. Kannan, Structure and mechanical properties of supercritical carbon dioxide processed porous resorbable polymer constructs, J. Mech. Behav. Biomed. Mater. 2 (2009) 620–626. doi:10.1016/j.jmbbm.2008.11.006.

[12] D. Mondal, M. Griffith, S.S. Venkatraman, Polycaprolactone-based biomaterials for tissue engineering and drug delivery: Current scenario and challenges, Int. J. Polym. Mater. Polym. Biomater. 65 (2016) 255–265. doi:10.1080/00914037.2015.1103241.

[13] S.A. Abbah, C.X.L. Lam, D.W. Hutmacher, J.C.H. Goh, H.K. Wong, Biological performance of a polycaprolactone-based scaffold used as fusion cage device in a large animal model of spinal reconstructive surgery, Biomaterials. 30 (2009) 5086–5093. doi:10.1016/j.biomaterials.2009.05.067.

[14] S.A. Abbah, C.X.F. Lam, K.A. Ramruttun, J.C.H. Goh, H.-K. Wong, Autogenous Bone Marrow Stromal Cell Sheets-Loaded mPCL/TCP Scaffolds Induced Osteogenesis in a Porcine Model of Spinal Interbody Fusion, Tissue Eng. Part A. 17 (2011) 809–817.

doi:10.1089/ten.tea.2010.0255.

[15] Y. Li, Z. gang Wu, X. kang Li, Z. Guo, S. hua Wu, Y. quan Zhang, L. Shi, S. hin Teoh, Y. chun Liu, Z. yong Zhang, A polycaprolactone-tricalcium phosphate composite scaffold as an autograft-free spinal fusion cage in a sheep model, Biomaterials. 35 (2014) 5647–5659. doi:10.1016/j.biomaterials.2014.03.075.

[16] M.R.N.O. Yong, S. Saifzadeh, G.N. Askin, R.D. Labrom, D.W. Hutmacher, C.J. Adam, Biological performance of a polycaprolactone-based scaffold plus recombinant human morphogenetic protein-2 (rhBMP-2) in an ovine thoracic interbody fusion model, Eur. Spine J. 23 (2014) 650–657. doi:10.1007/s00586-013-3085-x.

[17] R.J. Kroeze, T.H. Smit, P.P. Vergroesen, R.A. Bank, R. Stoop, B. van Rietbergen, B.J. van Royen, M.N. Helder, Spinal fusion using adipose stem cells seeded on a radiolucent cage filler: a feasibility study of a single surgical procedure in goats, Eur. Spine J. 24 (2015) 1031–1042. doi:10.1007/s00586-014-3696-x.

[18] P.-P.A. Vergroesen, R.-J. Kroeze, M.N. Helder, T.H. Smit, The Use of Poly(L-lactide-co-caprolactone) as a Scaffold for Adipose Stem Cells in Bone Tissue Engineering: Application in a Spinal Fusion Model, Macromol. Biosci. 11 (2011) 722–730. doi:10.1002/mabi.201000433.

[19] R.C. Thomson, M.J. Yaszemski, J.M. Powers, A.G. Mikos, Fabrication of biodegradable polymer scaffolds to engineer trabecular bone, J. Biomater. Sci. Polym. Ed. 7 (1995) 23–38. doi:10.1163/156856295X00805.

[20] V.M. Correlo, L.F. Boesel, E. Pinho, A.R. Costa-Pinto, M.L. Alves Da Silva, M. Bhattacharya, J.F. Mano, N.M. Neves, R.L. Reis, Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties, J. Biomed. Mater. Res. - Part A. 91 (2009) 489–504. doi:10.1002/jbm.a.32221.

[21] M.E. Gomes, H.S. Azevedo, A.R. Moreira, V. Ellä, M. Kellomäki, R.L. Reis, Starch-poly(epsilon-caprolactone) and starch-poly(lactic acid) fibre-mesh scaffolds for bone tissue engineering applications: structure, mechanical properties and degradation behaviour., J. Tissue Eng. Regen. Med. 2 (2008) 243–52. doi:10.1002/term.89.

[22] I.B. Leonor, M.T. Rodrigues, M.E. Gomes, R.L. Reis, In situ functionalization of wet-spun fibre meshes for bone tissue engineering, J. Tissue Eng. Regen. Med. 5 (2011) 104–111. doi:10.1002/term.294.

[23] A. Martins, A.R.C. Duarte, S. Faria, A.P. Marques, R.L. Reis, N.M. Neves, Osteogenic induction of hBMSCs by electrospun scaffolds with dexamethasone release functionality, Biomaterials. 31 (2010) 5875–5885. doi:10.1016/j.biomaterials.2010.04.010.

[24] I.F. Cengiz, M. Pitikakis, L. Cesario, P. Parascandolo, L. Vosilla, G. Viano, J.M. Oliveira, R.L. Reis, Building the basis for patient-specific meniscal scaffolds: From human knee MRI to fabrication of 3D printed scaffolds, Bioprinting. 1–2 (2016) 1–10. doi:10.1016/j.bprint.2016.05.001.

[25] J. Reignier, J. Tatibouët, R. Gendron, Batch foaming of poly(ε{lunate}-caprolactone) using carbon dioxide: Impact of crystallization on cell nucleation as probed by ultrasonic measurements, Polymer (Guildf). 47 (2006) 5012–5024. doi:10.1016/j.polymer.2006.05.040.

[26] S. Cotugno, E. Di Maio, G. Mensitieri, S. Iannace, G.W. Roberts, R.G. Carbonell, H.B. Hopfenberg, Characterization of Microcellular Biodegradable Polymeric Foams Produced from Supercritical Carbon Dioxide Solutions, Ind. Eng. Chem. Res. 44 (2005) 1795–1803. doi:10.1021/ie049445c.

[27] Q. Xu, X. Ren, Y. Chang, J. Wang, L. Yu, K. Dean, Generation of microcellular biodegradable polycaprolactone foams in supercritical carbon dioxide, J. Appl. Polym.

Sci. 94 (2004) 593–597. doi:10.1002/app.20726.

[28] I. Tsivintzelis, E. Pavlidou, C. Panayiotou, Biodegradable polymer foams prepared with supercritical CO2-ethanol mixtures as blowing agents, J. Supercrit. Fluids. 42 (2007) 265–272. doi:10.1016/j.supflu.2007.02.009.

[29] E. Kiran, K. Liu, K. Ramsdell, Morphological changes in poly(ε-caprolactone) in dense carbon dioxide, Polymer (Guildf). 49 (2008) 1853–1859. doi:10.1016/j.polymer.2008.02.017.

[30] A. Salerno, M.A. Fanovich, C.D. Pascual, The effect of ethyl-lactate and ethyl-acetate plasticizers on PCL and PCL-HA composites foamed with supercritical CO2, J. Supercrit. Fluids. 95 (2014) 394–406. doi:10.1016/j.supflu.2014.10.007.

[31] A. Salerno, E. Di Maio, S. Iannace, P.A. Netti, Solid-state supercritical CO2 foaming of PCL and PCL-HA nano-composite: Effect of composition, thermal history and foaming process on foam pore structure, J. Supercrit. Fluids. 58 (2011) 158–167. doi:10.1016/j.supflu.2011.05.009.

[32] A. Salerno, U. Clerici, C. Domingo, Solid-state foaming of biodegradable polyesters by means of supercritical CO2/ethyl lactate mixtures: Towards designing advanced materials by means of sustainable processes, Eur. Polym. J. 51 (2014) 1–11. doi:10.1016/j.eurpolymj.2013.11.015.

[33] A. Salerno, J. Saurina, C. Domingo, Supercritical CO2 foamed polycaprolactone scaffolds for controlled delivery of 5-fluorouracil, nicotinamide and triflusal, Int. J. Pharm. 496 (2015) 654–663. doi:10.1016/j.ijpharm.2015.11.012.

[34] T.-Y. Bak, M.-S. Kook, S.-C. Jung, B.-H. Kim, Biological Effect of Gas Plasma Treatment on CO 2 Gas Foaming/Salt Leaching Fabricated Porous Polycaprolactone Scaffolds in Bone

Tissue Engineering, J. Nanomater. 2014 (2014) 1–6. doi:10.1155/2014/657542.

[35] A. Salerno, S. Zeppetelli, E. Di Maio, S. Iannace, P.A. Netti, Design of bimodal PCL and PCL-HA nanocomposite scaffolds by two step depressurization during solid-state supercritical CO 2 foaming, Macromol. Rapid Commun. 32 (2011) 1150–1156. doi:10.1002/marc.201100119.

[36] E. Markočič, M. Škerget, Ž. Knez, Effect of Temperature and Pressure on the Behavior of Poly(ε-caprolactone) in the Presence of Supercritical Carbon Dioxide, Ind. Eng. Chem. Res. 52 (2013) 15594–15601. doi:10.1021/ie402256a.

[37] N. Annabi, A. Fathi, S.M. Mithieux, A.S. Weiss, F. Dehghani, Fabrication of porous PCL/elastin composite scaffolds for tissue engineering applications, J. Supercrit. Fluids. 59 (2011) 157–167. doi:10.1016/j.supflu.2011.06.010.

[38] M. Karimi, M. Heuchel, T. Weigel, M. Schossig, D. Hofmann, A. Lendlein, Formation and size distribution of pores in poly(??-caprolactone) foams prepared by pressure quenching using supercritical CO 2, J. Supercrit. Fluids. 61 (2012) 175–190. doi:10.1016/j.supflu.2011.09.022.

[39] M.A. Fanovich, P. Jaeger, Sorption and diffusion of compressed carbon dioxide in polycaprolactone for the development of porous scaffolds, Mater. Sci. Eng. C. 32 (2012) 961–968. doi:10.1016/j.msec.2012.02.021.

[40] E. Di Maio, G. Mensitieri, S. Iannace, L. Nicolais, W. Li, R.W. Flumerfelt, Structure optimization of polycaprolactone foams by using mixtures of CO2 and N2 as blowing agents, Polym. Eng. Sci. 45 (2005) 432–441. doi:papers://590F92D9-0B76-4B88-8729-9AF064BE5AC8/Paper/p2638.

[41] M.B.C. De Matos, A.P. Piedade, C. Alvarez-Lorenzo, A. Concheiro, M.E.M. Braga, H.C. De

Sousa, Dexamethasone-loaded poly(É-caprolactone)/silica nanoparticles composites prepared by supercritical CO2 foaming/mixing and deposition, Int. J. Pharm. 456 (2013) 269–281. doi:10.1016/j.ijpharm.2013.08.042.

[42] C. Marrazzo, E. Di Maio, S. Iannace, Conventional and nanometric nucleating agents in poly(??-caprolactone) foaming: Crystals vs. bubbles nucleation, Polym. Eng. Sci. 48 (2008) 336–344. doi:10.1002/pen.20937.

[43] D.L. Tomasko, H. Li, D. Liu, X. Han, M.J. Wingert, L.J. Lee, K.W. Koelling, A Review of CO_2 Applications in the Processing of Polymers, Ind. Eng. Chem. Res. 42 (2003) 6431–6456. doi:10.1021/ie030199z.

[44] A.R.C. Duarte, J.F. Mano, R.L. Reis, Dexamethasone-loaded scaffolds prepared by supercritical-assisted phase inversion, Acta Biomater. 5 (2009) 2054–2062. doi:10.1016/j.actbio.2009.01.047.

[45] A.R.C. Duarte, V.E. Santo, A. Alves, S.S. Silva, J. Moreira-Silva, T.H. Silva, A.P. Marques, R. a. Sousa, M.E. Gomes, J.F. Mano, R.L. Reis, Unleashing the potential of supercritical fluids for polymer processing in tissue engineering and regenerative medicine, J. Supercrit. Fluids. 79 (2013) 177–185. doi:10.1016/j.supflu.2013.01.004.

[46] A. Odgaard, Three-dimensional methods for quantification of cancellous bone architecture, Bone. 20 (1997) 315–328. doi:10.1016/S8756-3282(97)00007-0.

[47] L.M. Mathieu, T.L. Mueller, P. Bourban, D.P. Pioletti, R. Müller, J.-A.E. Månson, Architecture and properties of anisotropic polymer composite scaffolds for bone tissue engineering., Biomaterials. 27 (2006) 905–16. doi:10.1016/j.biomaterials.2005.07.015.

[48] L.J. Gibson, Biomechanics of cellular solids, J. Biomech. 38 (2005) 377–399. doi:10.1016/j.jbiomech.2004.09.027.

[49] L.J. Gibson, M.F. Ashby, Cellular solids: Structure and properties, 1990. doi:10.2277/0521499119.

[50] J. Ródenas-Rochina, J.L.G. Ribelles, M. Lebourg, Comparative study of PCL-HAp and PCL-bioglass composite scaffolds for bone tissue engineering, J. Mater. Sci. Mater. Med. 24 (2013) 1293–1308. doi:10.1007/s10856-013-4878-5.

[51] P.S.P. Poh, D.W. Hutmacher, B.M. Holzapfel, A.K. Solanki, M.M. Stevens, M.A. Woodruff, In vitro and in vivo bone formation potential of surface calcium phosphate-coated polycaprolactone and polycaprolactone/bioactive glass composite scaffolds, Acta Biomater. 30 (2016) 319–333. doi:10.1016/j.actbio.2015.11.012.

[52] C. Chen, L. Garber, M. Smoak, C. Fargason, T. Scherr, C. Blackburn, S. Bacchus, M.J. Lopez, J.A. Pojman, F. Del Piero, D.J. Hayes, *In Vitro* and *In Vivo* Characterization of Pentaerythritol Triacrylate-co-Trimethylolpropane Nanocomposite Scaffolds as Potential Bone Augments and Grafts, Tissue Eng. Part A. 21 (2015) 320–331. doi:10.1089/ten.tea.2014.0018.

[53] K. Rezwan, Q.Z. Chen, J.J. Blaker, A.R. Boccaccini, Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering, Biomaterials. 27 (2006) 3413–3431. doi:10.1016/j.biomaterials.2006.01.039.

[54] A.R. Boccaccini, J.A. Roelher, L.L. Hench, V. Maquet, R. Jrme, A Composites Approach to Tissue Engineering, in: Ceram. Eng. Sci. Proc., 2008: pp. 805–816. doi:10.1002/9780470294758.ch90.

[55] M. Navarro, A. Michiardi, O. Castan, J.A. Planell, Review. Biomaterials in orthopaedics, J. R. Soc. Interface. 5 (2008) 1137–1158. doi:10.1098/rsif.2008.0151.

[56] V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis,

Biomaterials. 26 (2005) 5474–5491. doi:10.1016/j.biomaterials.2005.02.002.

[57] Y. Zhou, D.W. Hutmacher, S.L. Varawan, T.M. Lim, In vitro bone engineering based on polycaprolactone and polycaprolactone-tricalcium phosphate composites, Polym. Int. 56 (2007) 333–342. doi:10.1002/pi.2138.

[58] N. Jaiswal, S.E. Haynesworth, a I. Caplan, S.P. Bruder, Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro., J. Cell. Biochem. 64 (1997) 295–312. doi:10.1002/(sici)1097-4644(199702)64:2<295::aid-jcb12>3.0.co;2-i.

[59] C. Correia, W. Grayson, R. Eton, J.M. Gimble, R.A. Sousa, R.L. Reis, G. Vunjak-Novakovic, Human adipose-derived cells can serve as a single-cell source for the in vitro cultivation of vascularized bone grafts, J. Tissue Eng. Regen. Med. 8 (2014) 629–639. doi:10.1002/term.1564.

Figure Captions

Figure 1. Schematic representation of the supercritical foaming equipment. BPR: Back Pressure Regulator, P: Pressure Transducer, TIC: Temperature Controller, FM: Flow Meter, CO_2: Carbon Dioxide.

Figure 2. Schematic representation of processing conditions (pressure and temperature) used in the work reported in the literature (circle symbol) as well as the conditions proposed herein (cross symbol).

Figure 3. Effect of time on the plasticization and foaming of polycaprolactone under dense carbon dioxide atmosphere at 45 °C.

Figure 4. Scanning electron microscopy (top row, scale bar: 500 μm) and micro-computed tomography images (bottom row) of the different formulations processed at 45 °C and 5.0 MPa.

Figure 5. Comparison of pore size of the PCL foams prepared in the literature (Table 1) and those described in this work (light grey – supercritical foaming conditions; dark grey – subcritical foaming conditions). Circle diameter represent the range of pore size reported in each work.

Figure 6. Micro-computed tomography 3D reconstruction images of the scaffolds produced at 45 °C, 5.0 MPa.

Figure 7: Strain-stress curves obtained in compression mode for the 3D constructs PCL and PCL + β-TCP, compared with a typical stress-strain curve as Example. Bottom left: A close-up of the elastic regime area.

Figure 8. Water uptake of the scaffolds (A) and pH of the solution (B) as a function of time for the scaffolds PCL (closed symbols) and PCL + β-TCP (open symbols)

Figure 9. In vitro release profile of the samples loaded with 5 wt% dex (open symbols) and 10 wt% dex (closed symbols), represented as Cumulative drug released (mg) (A) or Percentage of drug released (%). Bottom left: A close-up of the early time-points.

Figure 10. Cytotoxicity assay: cell viability (%) determined by indirect contact.

Fig 1

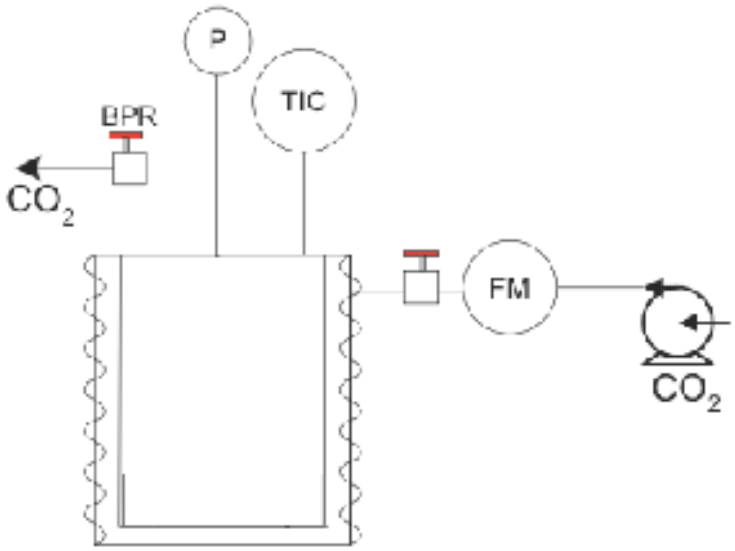

Fig 2

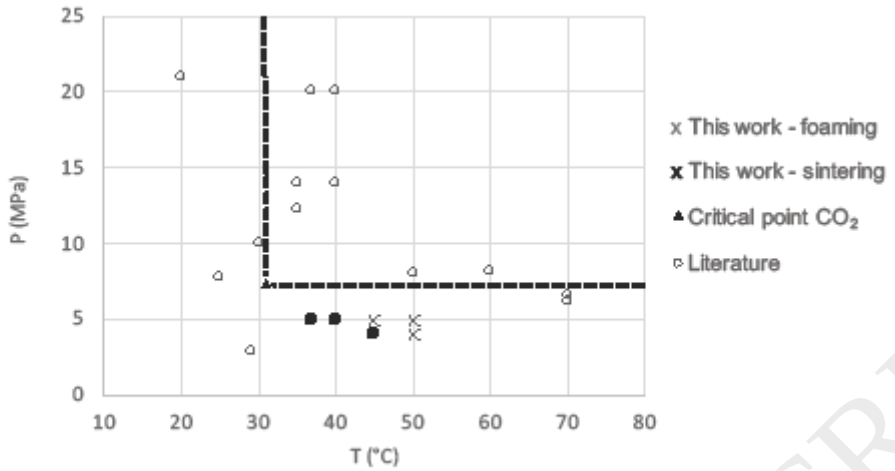

Fig 3

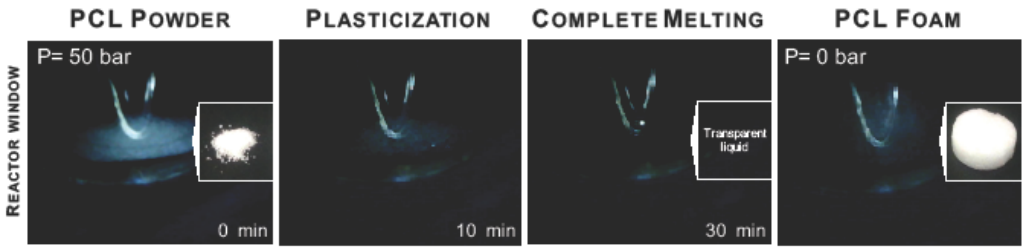

Fig 4

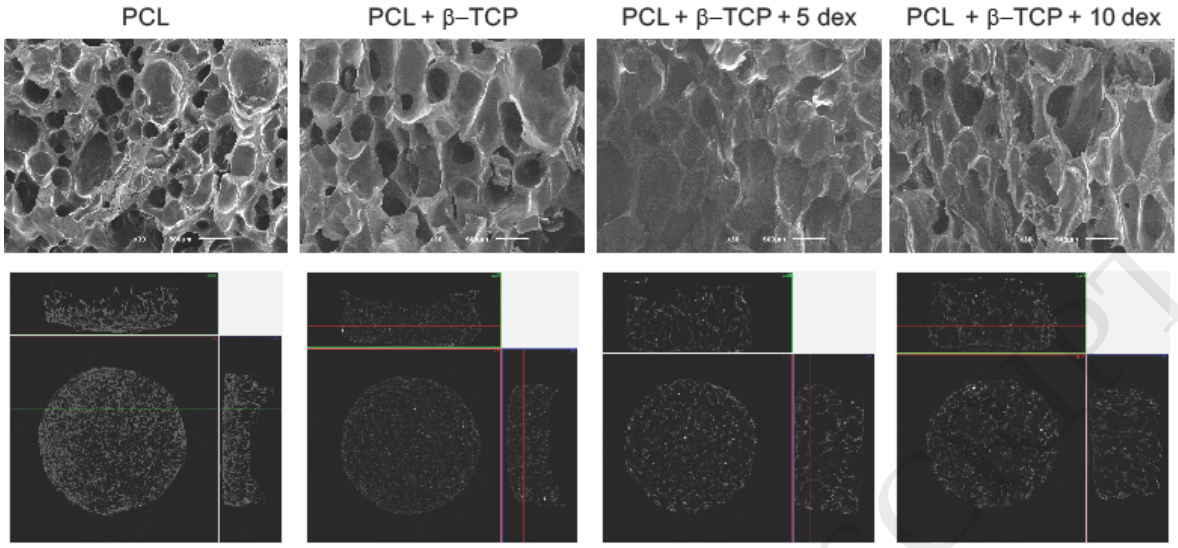

Fig 5

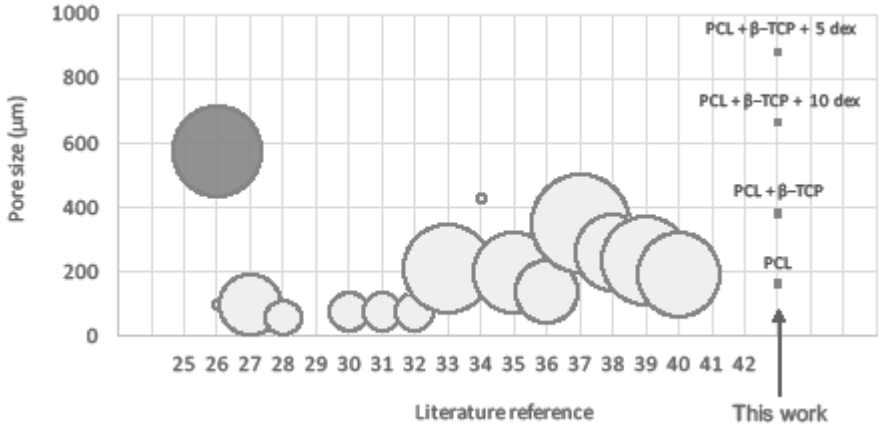

Fig 6

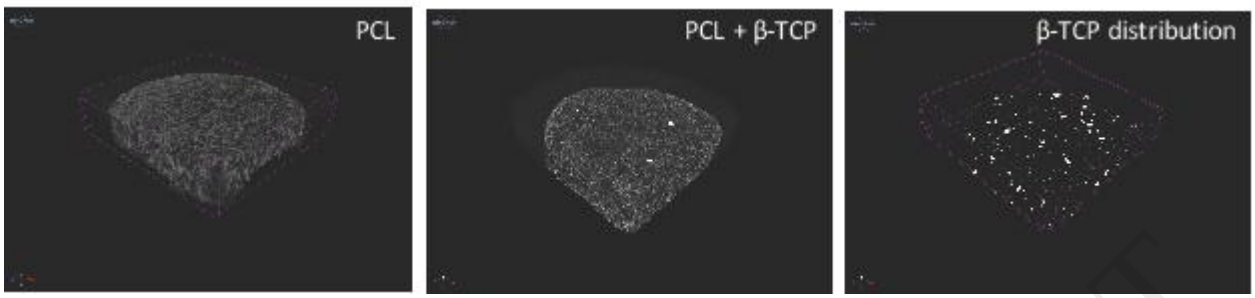

Fig 7

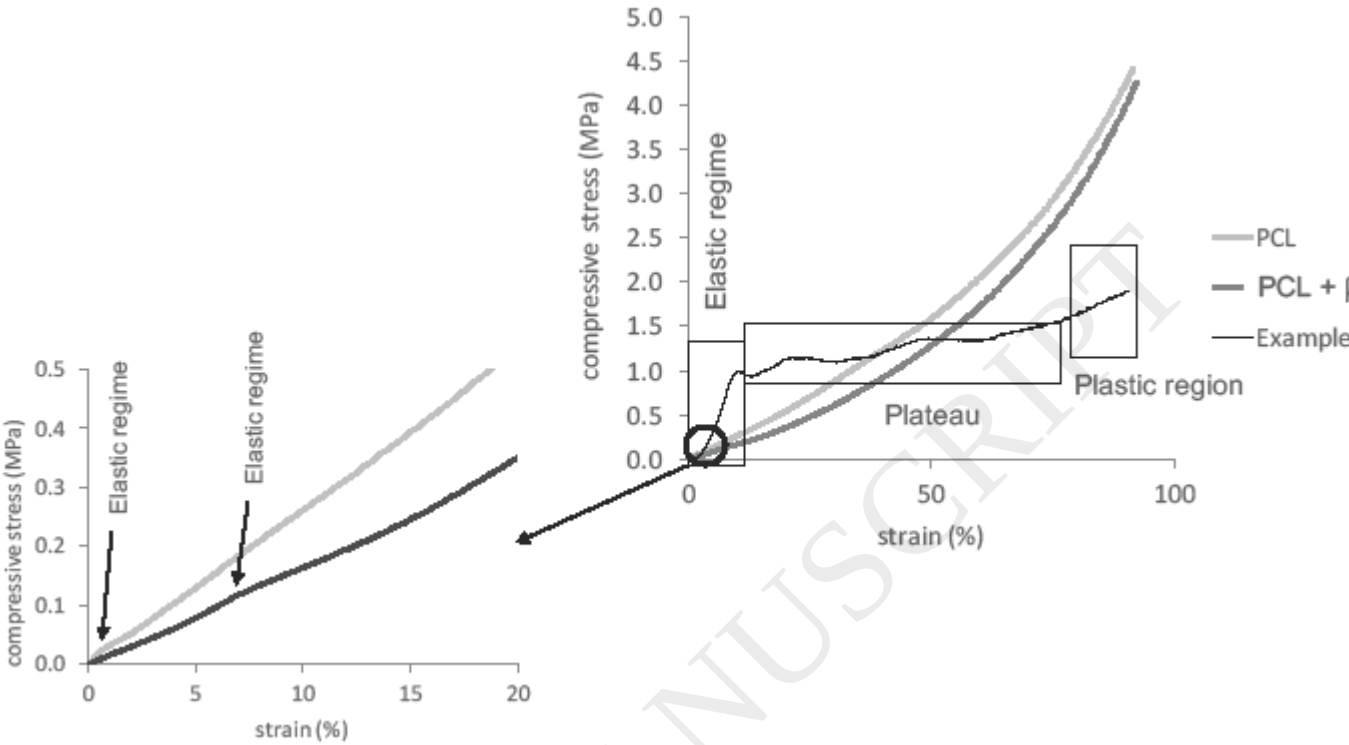

Fig 8

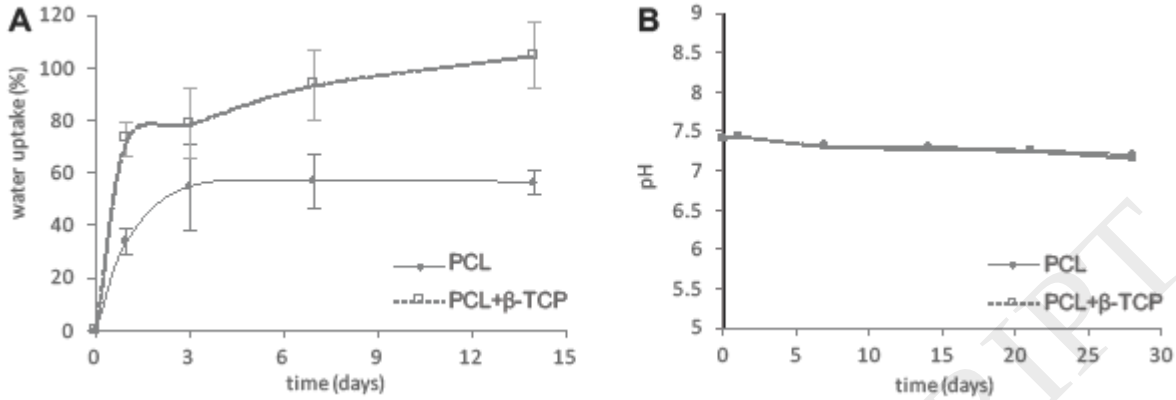

Fig 9

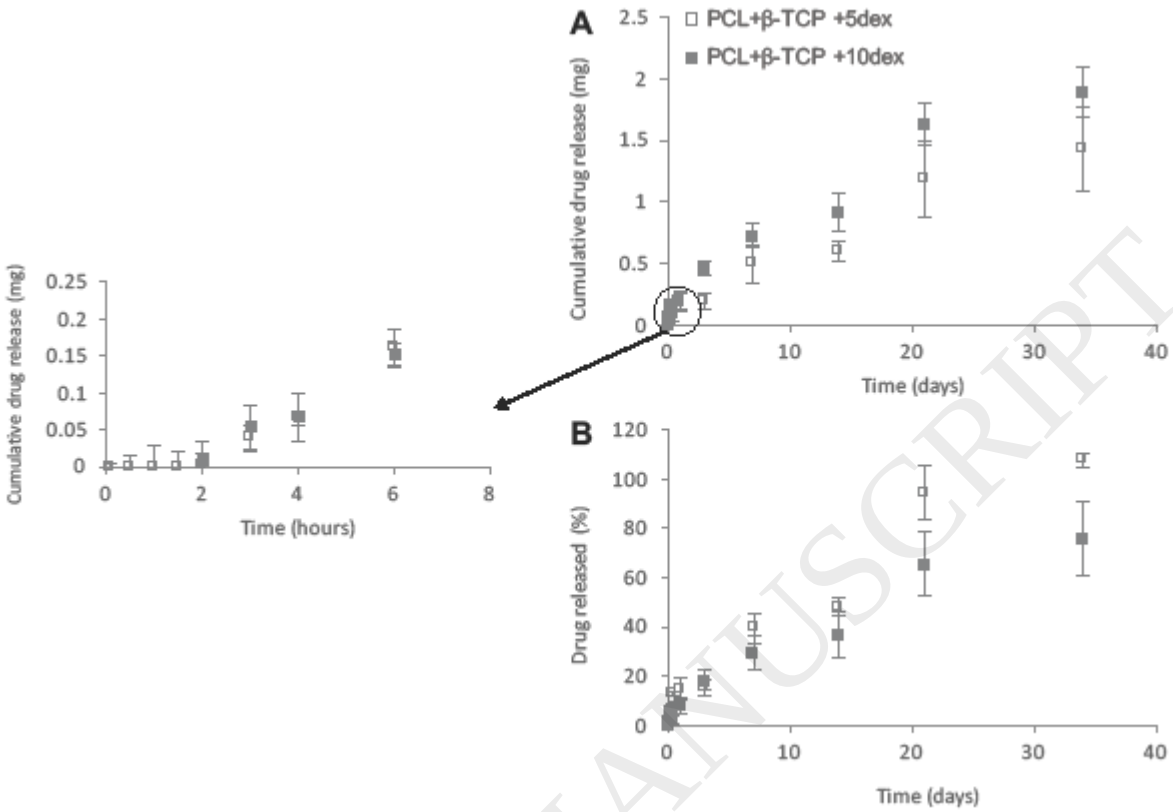

Fig 10

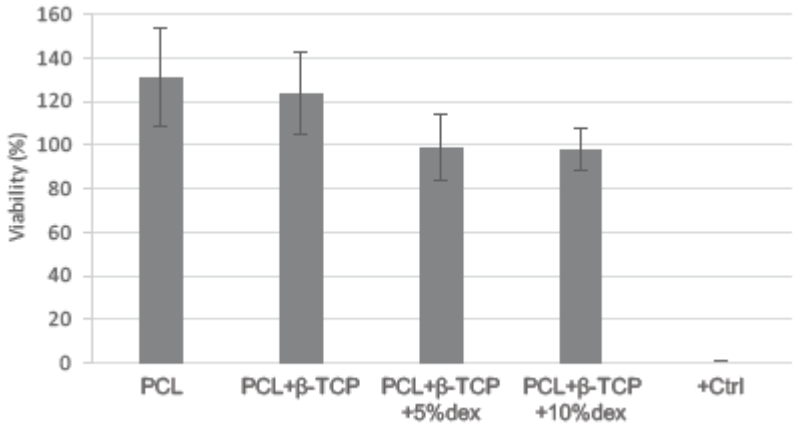

Table 1. Summary of the supercritical fluid studies on foaming of polycaprolactone reported in the literature.

	Operating parameters			Morphological properties		Mechanical properties	
Additives	**Pressure range**	**Temperature range**	**Molecular weight**	**Porosity**	**Pore size**	**Compressive modulus**	**Reference**
	(MPa)	**(°C)**	**(Da)**	**(%)**	**(μm)**	**(MPa)**	
Talc	6.2	70	-	Data not shown	Data not shown	Data not shown	[25]
	6.9*	24-30**	80 000	Data not shown	100 Pin > Pc CO2 450 - 700 Pin < Pc CO2	Data not shown	[26]
	8.0-16.0	40	85 000	Data not shown	40 -160	Data not shown	[27]
Ethanol	12.3-20.5	35-45	80 000	Data not shown	40 - 80	Data not shown	[28]
	21.0-45.0	20-50	14 000	Data not shown	Data not shown	Data not shown	[29]
Ethyl lactacte	20.0	40-45	80 000	65-80%	50 -200	Data not shown	[30–32]
5-fluorouracil, nicotinamide and triflusal	20.0	40	80 000	61-74%	87-337	Data not shown	[33]
NaCl	8.0	50	30 -50 000	Data not shown	427	Data not shown	[34]
HA	20.0	37	65 000	~90%	50-70 and 100-300	Data not shown	[35]
	20.0-30.0	40-60	80 000	Data not shown	80-200	Data not shown	[36]
Elastin + NaCl	6.5	70	80 000	70-90 %	200-500	Data not shown	[37]
	7.8-20.0	25-50	69-120 000	65-90 %	170-350	Data not shown	[38]
	10.0-20.0	30-45	74 000	Data not shown	110-360	Data not shown	[39]

	2.8-5.9	29-45**	80 000	Data not shown	80-300	Data not shown	[40]
Mesoporous silica particles Dexamethasone +	14.0-25.0	35	40 000	20-50%	no macroporosity evaluated	20-250	[41]
	8.2	60	70 000	Data not shown	Data not shown	Data not shown	[42]

*Psat bewteen 6.9 and 32 MPa **T sat = 70ºC

Table 2: Summary of the different formulations prepared in this work (PCL (Mn 45000 Da) mass was kept constant in all formulations, 500 mg were used).

#	Designation	β-TCP (wt %)	Dexamethasone (wt %)
1	PCL	-	-
2	PCL + β-TCP	10	-
3	PCL + β-TCP + dex5	10	5
4	PCL + β-TCP + dex10	10	10

Table 3. Morphological characterization of the scaffolds by microCT.

	PCL	**PCL + β–TCP**	**PCL + β–TCP + 5 dex**	**PCL + β–TCP + 10 dex**	**Trabecular bone** [47]
Porosity (%)	73	90	98	84	52-96
Pore Interconnectivity (%)	79	97	99	95	-
Mean pore size (μm)	164	383	882	664	450-1310
Density (mm^{-1})	28	54	66	31	7-34
Degree of anisotropy	1.42	1.48	1.38	1.48	1.1-2.38