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Rouholamin, D., van Grunsven, W., Reilly, G.C. et al. (1 more author) (2016) Morphological effects of porous poly-D,L-lactic acid/hydroxyapatite scaffolds produced by supercritical CO2 foaming on their mechanical performance. Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine, 230 (8). pp. 761-774. ISSN 0954-4119

https://doi.org/10.1177/0954411916650221

Morphological effects of porous $P_{DL}LA$/HA scaffolds produced by supercritical CO_2 foaming on their mechanical performance

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Abstract

A novel supercritical CO_2 foaming technique was used to fabricate scaffolds of controllable morphology and mechanical properties, with the potential to tailor the scaffolds to specific tissue engineering applications. Biodegradable scaffolds are widely used as temporary supportive structures for bone regeneration. The scaffolds must provide a sufficient mechanical support while allowing cell attachment and growth as well as metabolic activities. In this study, supercritical CO_2 foaming was used to prepare fully interconnected porous scaffolds of poly(D,L)lactic acid and poly(D,L)lactic acid/hydroxyapatite. The morphological, mechanical and cell behaviours of the scaffolds were measured to examine the effect of hydroxyapatite on these properties. These scaffolds showed an average porosity in the range of 86-95%, an average pore diameter of 229-347 µm and an average pore interconnection of 103-207 µm. The measured porosity, pore diameter and interconnection size are suitable for cancellous bone regeneration. Compressive strength and modulus of up to 36.03 ± 5.90 MPa and 37.97 ± 6.84 MPa were measured for the produced porous scaffolds of various compositions. The mechanical properties presented an improvement with the addition of hydroxyapatite to the structure. The relationship between morphological and mechanical properties was investigated. The matrices with different compositions were seeded with bone cells, and all the matrices showed a high cell viability and biocompatibility. The number of cells attached on the matrices slightly increased with the addition of hydroxyapatite indicating that hydroxyapatite improves the biocompatibility and proliferation of the scaffolds. The produced poly(D,L)lactic acid/hydroxyapatite scaffolds in this study showed a potential to be used as bone graft substitutes.

Keywords

Biodegradable, compression, foams, porosity, supercritical CO_2

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Introduction

Bone is a complex vascularised tissue with a capability to heal, reconstruct and mobilise mineral. This natural composite material possesses a unique capacity to bear functional load and protect internal organs. Therefore, any disease, injury or disfigurement of bone structure can cause major health problems for the body.[1] The main constituent of a bone is a porous cellular structure of cancellous or trabecular bone surrounded by a dense outer shell of compact or cortical bone. Cancellous bone minimises the weight of bone while maintaining mechanical function.[2] Organ or tissue transplantation has been recognised as a standard way to treat patients suffering organ disease or tissue loss. However, there are limited numbers of willing donors.[3] Pathogen transfer and immune rejection are the other disadvantages of this type of therapy, and therefore, tissue engineering and the use of artificial bone grafts have been recently considered as an alternative approach to overcome these limitations.[4] Tissue engineering involves seeding and growing cells in three-dimensional biodegradable scaffolds to form and regenerate new organs or tissues.[3-5]

A biodegradable scaffold needs to act as a temporary template for cell proliferation and growth leading to tissue formation. The matrix must possess several characteristics to become an appropriate structure for bone regeneration purposes. Suitable choice of biomaterial and sufficient morphological and mechanical properties are required for successful bone regeneration. The structure of the scaffold needs to allow bone cell attachment and growth as well as transfer of oxygen, nutrients and metabolic waste. Hence, the scaffold needs to possess a highly porous structure with fully interconnected pores.[1,6] The majority of studies in this field claim that pore size needs to be within 200-500 μm.[3,4,10-13] Higher surface area to volume ratios can be obtained with larger pores and higher degrees

of porosity, which may facilitate bone tissue growth and metabolic activities. However, the mechanical stability of the biodegradable scaffold may be adversely affected; limiting its range of application. Thus, the degree of porosity and pore size must be balanced with the mechanical requirements of the target tissue. The mechanical properties of the implanted scaffolds should be sufficient to bear the local load in the region in which the implant is placed and to maintain the space for tissue growth and metabolic activity. Furthermore, the scaffold needs to maintain its mechanical functionality until the new bone tissue is entirely regenerated and reconstructed.[1]

Various kinds of biomaterials including ceramics, metals and polymers have been used for tissue or organ implantation. Each of these types of biocompatible materials possesses its advantages and disadvantages.[1,3,14] Although, metals provide sufficient mechanical support at the site of implantation, they present poor integration with the surrounding tissue. Ceramics exhibit excellent biocompatibility and osteoconductivity. However, they show low toughness and tensile strength. Thus, ceramic biomaterials cannot be used in sites under high bending, torsion or shear stress.[1,15] Polymeric biomaterials have been extensively used for tissue engineering applications due to their controllable biodegradability. Both natural and synthetic polymers can be used in tissue engineering applications. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL) have received significant attention, and been used for various bone regeneration applications.[3]

PLA in the form of poly-L-lactic acid (P_LLA) and poly-D,L-lactic acid ($P_{DL}LA$) has been widely used as a polymer biomaterial for preparation of temporary implants in tissue engineering. These materials possess high toughness and are biodegradable. However, the degradation products of these materials reduce the local pH value which accelerates the

scaffold degradation rate and can cause tissue inflammation. In addition, PLA possesses low compressive strength which limits its application in bone regeneration.[4,12,15]

Hydroxyapatite (HA) has been extensively used as a ceramic biomaterial for bone regeneration due to its high biocompatibility, nontoxicity and excellent osteoconductive properties. However, the low toughness of HA limits its use as a load bearing scaffold for tissue engineering.[4,15]

Recently, PLA/HA composite materials have received increased interest in tissue engineering and bone regeneration. These composite materials present a better environment for cell seeding and growth and improved integration with surrounding tissues due to the high biocompatibility and osteoconductive properties of HA. HA buffers the acidic by-products generated during the degradation process, and therefore provides a controlled degradation rate for the composite material. These composite materials show improved structural integrity and mechanical properties for tissue engineering compared to pure PLA or HA materials.[4,15,16]

There are several manufacturing techniques available to prepare porous biodegradable scaffolds. The processing technique needs to be selected according to the morphological and mechanical needs for the scaffolds without adversely affecting the properties of the chosen biomaterial.[1,3,14] The fabrication method must be consistent and precise with regards to pore size and structure. Supercritical CO_2 ($scCO_2$) foaming technique as a solvent-free process has been used for several years for preparing porous $P_{DL}LA$, P_LLA, PLGA and PGA scaffolds. $ScCO_2$ is carbon dioxide at or above its critical pressure (73.8bar) and temperature (31.1°C). The diffusivity of a gas and the density and dissolving power of a liquid with the relatively low critical point for $scCO_2$ propose a popular choice for fabricating porous structures.[12,15,17] In this technique, polymer or polymer composite disks are equilibrated with $scCO_2$ which

diffuses into the polymer structure and lowers the glass transition temperature (T_g) of the polymer. Polymer is plasticised, and a solution of polymer with CO_2 is formed. A thermodynamic instability is obtained by rapidly reducing CO_2 pressure to ambient pressure. As a result of this rapid drop in pressure, T_g begins to rise and CO_2 escapes from the polymer phase causing the nucleation of bubbles and the fabrication of foams. Bubbles are expanded by the penetration of an increasing amount of gas, and the polymer scaffold is expanded rapidly due to pore growth. As a result of the reduction in pressure and temperature, the viscosity of the polymer is increased, and the foam architecture is progressively fixed. Hence, a porous polymer or polymer composite foam is formed. The desirable porous structure can be achieved in only 1 to 3 hours when $scCO_2$ is used, however, 24 to 72 hours is required to prepare porous scaffolds using CO_2.[1,3,4,10,12,13,15]

Absence of high temperatures or organic solvents and high speed processing where $scCO_2$ is used as the foaming gas are some of the advantages provided by $scCO_2$ foaming compared to other processing techniques available in this field.[15,18] However, some disadvantages have been reported for this technique. Poor interconnectivity between pores is one of these disadvantages, and has an adverse effect on the cell growth and metabolic activities.[1,3,13,15] Low mechanical strength has also been reported for scaffolds prepared with this technique which makes these scaffolds inappropriate for mechanically demanding areas in tissue implantation.[1]

In this study, we aimed to create porous composite scaffolds with improved mechanical properties compared to the $P_{DL}LA$ scaffold. We previously developed a novel $scCO_2$ foaming technique to fabricate the porous polymer structures. The effects of various processing conditions on the morphology of porous $P_{DL}LA$ scaffolds were studied and investigated in a previous study.[19]

Experimental section

Materials

$P_{DL}LA$ (Purac, The Netherlands) in granular form with a molecular weight of 406 000 g/mol was used as received. The weight average molecular weight of the polymer was measured using Gel Permeation Chromatography in chloroform at 35 °C relative to polystyrene standards. The T_g of the polymer was measured at 60-65 °C using Differential Scanning Calorimetry. Ball-milled HA particles (Plasma Biotal Limited, United Kingdom) with an average size of 1-2 µm and specific area of 20-30 m^2/g measured by Brunauer-Emmett-Teller method was added to $P_{DL}LA$. Food grade CO_2 (BOC, United Kingdom) with 99% purity was used as a foaming agent without further purification.

Polymer and polymer composite disk fabrication

In the current study, 2 and 4 wt% HA were added to $P_{DL}LA$ particles to prepare composite scaffolds. The weight fraction of HA was based on the total mass of $P_{DL}LA$ and HA. The $P_{DL}LA$/HA compositions were dry mixed using an electrical shaker (Turbula System Schatz, Switzerland) before being transferred to cylindrical stainless steel moulds with diameter of 19 mm. The moulds were then closed and compressed using a Hydraulic press (Specac, United Kingdom) at 130 MPa for 3 min in order to remove any air trapped between the particles. Disks were subsequently heated in a vacuum oven at 180˚C for 2 hours. This temperature is suitable for bonding between polymer particles to occur without degradation of the polymer. After cooling, disks were removed from the moulds and stored in a vacuum desiccator. Polymer and polymer composite scaffolds with various compositions before and after foaming are shown in Figure 1. The scaffolds are 19 mm in diameter and 4.5 mm thick.

[insert Figure 1.]

Porous scaffolds preparation by $scCO_2$ foaming technique

The $P_{DL}LA$ disks were placed into a sealed stainless steel pressure vessel, which was equipped with pressure and temperature controllers for monitoring the process. The vessel was pressurised to a desired pressure of 140 bar called 'saturation pressure', and the temperature was increased up to a desired temperature of 40 °C called 'saturation temperature' over a period of filling time. After filling the vessel with CO_2, the mixture of the polymer and CO_2 was maintained at constant pressure and temperature for 90 minutes known as 'saturation time'. The vessel was depressurised after saturation to ambient pressure, and temperature was decreased to ambient temperature over a period of 5 minutes venting time. Expanded porous scaffolds with a thin layer of nonporous skin and size of 20-26 mm diameter and 15-30 mm height were produced by this process.

In order to perform surface porosity studies, the nonporous skin of the scaffolds was removed with a razor blade after the scaffolds had been submerged in liquid nitrogen for 2 minutes and the cut surfaces coloured by a marker pen (Figure 2). Similar foaming parameters were used to prepare polymer and composite foams which enabled a direct comparison of morphology to be made between different compositions regardless of processing conditions. Various foaming conditions were performed for neat $P_{DL}LA$ to study the effect of foaming parameters on the morphological properties of the scaffolds. Pore nucleation and growth are the key factors in a gas foaming technique that affect pore structure and size. These two factors are mainly determined by the amount of CO_2 that is dissolved in the polymer structure and by the CO_2 diffusion and depressurisation rates. Other foaming parameters which can affect the porous structure of the scaffolds include the scaffold composition and saturation time.

[insert Figure 2.]

In this foaming method, the temperature needs to be selected above the critical temperature (31.1°C) and below the glass transition temperature of $P_{DL}LA$ (60-65°C) to use $scCO_2$ and prepare suitable porous structures. Various foaming conditions which were performed for $P_{DL}LA$ scaffolds to study the influence of foaming parameters on the morphological properties of the scaffolds are presented in Table 1.

Table 1. Processing conditions of $scCO_2$ foaming.

Saturation pressure (bar)	100	120	140	160	180
Venting time (min)	1	5	10	----	----
Saturation time (min)	30	60	90	120	----
Saturation temperature (°C)	32	40	50	----	----

Morphological characterisation

Optical microscopy was used to determine the surface pore size of the scaffolds. The surfaces of three scaffolds were characterised for each processing condition or composition. Average surface pore diameter for different scaffolds was estimated by the linear intercept method.[20] Micro computed tomography (micro-CT) was used for 3D morphological characterisation.[11] 3D morphology characterisations were performed using Skyscan 1172 with the following scanning conditions: a current of 167µA, voltage of 60kV, pixel size of 4.3 µm, 360˚ rotation, 0.7˚ rotation step. Scaffolds were scanned, and 3D analysis was performed on four different regions of interest in the 3D structure using CTAn software. The CT analysis was performed to estimate the mean value for architectural characteristics including porosity, average pore size and wall thickness, pore size and wall thickness distributions, interconnectivity, size of

pore interconnections, pores in the pore walls and degree of anisotropy. 3D models from micro-CT scans for various scaffolds were created using CTVol software. The level of interconectivity between the pores was measured using micro-CT analyses. This was carried out by measuring the volume of open and closed pores in the scaffolds by CT scanning. The interconnectivity was then measured by micro-CT 3D analysis using Equation (1):

Interconnectivity = (volume of open pores / sum of volume of open and closed pores) × 100% (1)

A porous structure consists of irregularly shaped voids and connecting channels or pore interconnections. Pore interconnections, often called fenestrations, are formed in the structure of the scaffolds as a result of pores merging together. Merging pores and presence of interconnections can be an evidence for interconnectivity of the pores in the structure. Pore interconnections as well as porosity, pore size and interconnectivity play a key role in cells expansion and migration.[16,21-23] Cells can be distributed uniformly and grow and expand readily where the pore interconnections are sufficiently large in an interconnected porous structure. The size of the interconnections were measured in this study by randomly selecting 10 cross sections from the 3D structure of each scaffold and measuring the size of the interconnections in those cross sections using ImageJ software. Three scaffolds from each composition were considered for the measurements of pore interconnections.

Surface roughness in the structure of the scaffolds is an important factor that can influence cell behaviour. Investigation and measurement of the pores on the surface of the pore walls was performed in order to assess the roughness of these surfaces in the structure. The surface roughness can improve cell adhesion, and modulate the biological activity of tissue where in contact with implants.[21]

Anisotropy is a measure of nonuniformity in the scaffold strut alignment.[11] Cancellous bone possesses an anisotropic architecture. Several methods have been introduced to measure the structural anisotropy of cancellous bone. The eigenvalue, which provides information about the direction of the axes in the ellipsoid in the structure of scaffold, was calculated using micro-CT. Degree of anisotropy can be mathematically determined using Equation (2):

$$\text{Degree of anisotropy} = 1 - (\text{min eigenvalue} / \text{max eigenvalue}) \quad (2)$$

Where degree of anisotropy is equal to 0 for fully isotropic and is equal to 1 for fully anisotropic structure.[24]

Scanning Electron Microscopy (SEM) was also used in order to investigate the cellular morphologies of the 3D structures. The scaffolds were freeze fractured in liquid nitrogen and sputter coated with gold for 3 min under an argon atmosphere at a current rate of 15 mA in an emscope SC 500 unit. These scaffolds were then visualised using SEM with a FEI Inspect F scanning electron microscope (10 kV). Different magnifications were employed to assess the pore shape and the microstructure of the pore walls. Similar to micro-CT, SEM investigation was performed on three scaffolds from each composition.

Mechanical characterisation

Mechanical strength of the porous scaffolds with various compositions was obtained and compared through compression testing using a compression test machine (Hounsfield, UK) with a crosshead speed of 0.5 mm/min. Cylindrical scaffolds of neat PLA and PLA/HA composite with 13 mm thickness and 20 mm diameter were used to investigate the mechanical properties of the scaffolds. Five specimens with parallel surfaces, perpendicular to the compression testing direction were prepared and tested for each composition. The ratio

of thickness to diameter was 0.65 in this study, which was smaller than the suggested ratio of 1 for an ideal compression test. Hence, the results of the test might have been slightly affected by the friction between the grips of compression test machine and the surface of the specimen. This has been taken into account in the analysis of the results. A slight variation in diameter and thickness of the porous scaffolds was noted, and recorded for the testing.

Biocompatibility test

In order to examine the biocompatibility of the scaffolds and the effect of addition of HA to the structure on the cell viability, scaffolds with various compositions were seeded with human osteosarcoma cells (MG-63) in vitro. The scaffolds need to be of the similar size, surface area and thickness for cell culture purposes. Disc-shaped matrices of various compositions with a uniform 1 mm thickness and approximately 20 mm diameter were sliced off by a razor blade after freezing the scaffolds in liquid nitrogen for 2 min. Scaffolds were sterilised by peracetic acid (PAA). 1×10^6 MG-63 were passaged and cultured in high-glucose DMEM supplemented with 10% FCS (Fetal Calf Serum, Biosera), 1% pen/strep solution, 1% L-glutamine. Cells were trypsinised and suspended in culture medium. 80 000 cells in 100 µL were seeded on each scaffold and cultured in a humidified cell culture incubator at 37°C with 5% Co_2; media was replaced every 3-4 days. Viability of the cells was examined using a resazurin assay of cell metabolic activity on day 1, 4 and 7. Resazurin measures the metabolic activity of the cells by measuring their ability to chemically reduce the intracellular environment. The fluorescence of resazurin is increased when it is metabolised by cells. The cell culture media was removed and fresh medium containing resazurin was added to the scaffolds and incubated. The solution was then removed and fluorescence intensity was measured using Microplate Fluorescence Reader (Bio-Tek Instruments, United States) and KC4 Data Analysis Software (Bio-Tek Instruments).

A fluorescence microscope (ImageXpress, Molecular Devices Ltd, United Kingdom) was used as an automated cellular imaging and analysis system to observe how seeded cells were distributed on the scaffolds. Cells were fixed and stained with Phalloidin-TRITC to stain the F-actin of the cell cytoskeleton red, and DAPI to stain the cell nuclei blue. Several images were captured from various areas of the scaffolds. Phalloidin-TRITC was visualised using 543 nm excitation wavelength, and DAPI was visualised using 461 nm excitation wavelength.

In this study, four scaffolds were seeded with human osteoblast-like cells (MG-63) for each composition. The statistical analysis (Tukey's pair-wise comparison, $p<0.05$) were carried out on the obtained results.

Results and discussion

Morphology study

Both surface and internal structure of the porous scaffolds were studied using optical microscopy and micro-CT respectively to investigate the effects of various compositions and foaming conditions on the pore size and structure. The average surface pore size and 3D morphological parameters of pure $P_{DL}LA$ and $P_{DL}LA$/HA composite scaffolds with different amounts of HA in the structure are reported in Table 2. Pore diameter distribution (Figure 3a) and pore wall thickness distribution (Figure 3b) in the 3D morphology of the scaffolds were also measured using micro-CT analysis. The images of the surface of $P_{DL}LA$ and $P_{DL}LA$-4 wt% HA obtained by optical microscope and images of the cross section of these scaffolds obtained by micro-CT are shown in Figure 4. The SEM images of the 3D structure of a $P_{DL}LA$ scaffold are also presented in Figure 4.

Table 2. 3D morphology properties of scaffolds with different compositions.

Scaffold composition	Porosity (%)	Pore interconnectivity (%)	Surface average pore diameter (µm)	Average pore diameter in the 3D structure (µm)	Average pore wall thickness in the 3D structure (µm)	Degree of anisotropy
Neat $P_{DL}LA$	92.39 ± 1.2	99.99	392.99 ± 71.30	346.96 ± 14.87	15.83 ± 0.84	0.51 ± 0.04
$P_{DL}LA$-2 wt% HA	90.69 ± 4.3	99.99	261.06 ± 52.03	242.71 ± 34.61	18.12 ± 2.13	0.79 ± 0.05
$P_{DL}LA$-4 wt% HA	89.47 ± 3.9	99.99	245.99 ± 64.64	229.40 ± 28.39	20.62 ± 1.07	0.97 ± 0.08

[insert Figure 3.]

[insert Figure 4.]

As it is presented in Table 2, pore size both on the surface and in the 3D morphology was in the range of 200-500 µm for the scaffolds produced using different compositions. The porosity was measured at 86-94% for different scaffolds using micro-CT (Table 2). These values of pore size and porosity are suitable for cancellous bone regeneration applications.[7-10] A decrease in the average surface and 3D morphology pore sizes and an increase in pore wall thickness were observed by addition of HA to the structure. The porosity of the scaffolds was lower in scaffolds containing HA.

The smaller pore size and porosity in scaffolds with HA can be due to the increase in the viscosity of the matrix and the decrease of CO_2 diffusion into the matrix following the addition of HA to the structure. This can lead to limited foam expansion and the formation of scaffolds with lower porosity and thicker pore walls.[4] This also results in formation of pores with the pore diameter and wall thickness distributions shown in Figure 3. As indicated, majority of the pores for neat $P_{DL}LA$ are in the range of 269-402 µm and no pores in the

range of 4-137 µm and majority of the pore walls are in the range of 13-22 µm. However for P_{DL}LA-2 wt% HA and P_{DL}LA-4 wt% HA scaffolds, the majority of the pores possess diameters in the range of 137-269 µm, a considerable number of pores in the range of 4-137 µm and smaller number of the pore walls possess thickness in the range of 13-22 µm. Therefore, the decrease in the pore size and increase in pore wall thickness with the addition of HA to the structure can also be observed in the pore diameter and wall thickness distributions. The decrease in pore size and increase in pore wall thickness with the addition of HA both on the surface and in the 3D morphology can also be seen in optical microscopy (Figure 4a and b) and micro-CT images (Figure 4c and d).

As mentioned earlier, the morphological properties of the scaffolds measured by micro-CT with 4.3 µm resolution, and thus it is possible that there were pores or pore walls smaller than 4.3 µm in the structure of the scaffolds which were not measured in the CT analysis. However, this cannot be a problem since same CT machine with same resolution was used in this study for all the scaffolds, and the aim of was to compare the results for scaffolds with different compositions.

It can be observed from the SEM images in Figure 4e and f that the scaffold possesses a highly porous network providing a high surface area suitable for cell attachment. The large surface area for cell attachment is shown in Figure 4f which indicates the structure of the pore walls. It can be seen that the pore walls contain a homogeneous and continuous phase of P_{DL}LA.

The interconnectivity between pores was measured at 99.99% for all scaffolds produced with various compositions and foaming conditions (Table 2). This open pore structure benefits cell culture and uniform distribution of seeded cells throughout the polymer or composite matrix.[19] However, several studies[3,13,15] have reported that the pressurised

CO_2 foaming technique results in the formation of a closed cellular structure and gives poor interconnectivity between the pores, which leads to a nonuniform distribution of cells throughout the scaffold. These types of scaffold also have an adverse effect on the transfer of nutrition and waste in the scaffold. The high interconnectivity between the pores obtained in the current study can be due to the high diffusion rate and solubility of $scCO_2$ into the structure of the scaffolds.[25]

The presence of interconnections and their size is important for cell adhesion and growth. Interconnections mainly appear where pores are merging together. Fig. 5 shows an example of these merging pores for a $P_{DL}LA$ scaffold. The area highlighted with a red circle shows a part of the scaffold where a number of pores merge together and form a larger pore.

[insert Figure 5.]

The sizes of the small pores in the walls were therefore measured in this study via randomly selecting 10 cross sections from the 3D structure and measuring the size of the pores in the walls of four areas from each cross section. Fig. 6a shows an image of a cross section of a $P_{DL}LA$ scaffold with a highlighted pore which is magnified in Fig. 6b. Fig. 6c indicates a number of the measured pore interconnections for a $P_{DL}LA$ scaffold.

[insert Figure 6.]

Table 3 presents the average size measured for the pore interconnections and for the small pores in the walls of scaffolds with different compositions.

Table 3. Size of pore interconnections and pores in the pore walls for scaffolds with different compositions.

Scaffold composition	Pore interconnections (μm)	Pores in the pore walls (μm)

Neat $P_{DL}LA$	207.28 ± 116.11	15.63 ± 8.69
$P_{DL}LA$-2 wt% HA	138.44 ± 67.83	11.89 ± 6.35
$P_{DL}LA$-4 wt% HA	102.56 ± 66.71	10.27 ± 6.22

The typical size of bone cells has been reported to be 20-30 µm.[26] As presented in Table 3, the size of pore interconnections is much larger than the size of individual cells for all scaffolds with different compositions, and this can allow the cells to migrate from one pore to another and throughout the scaffolds. Lu et al. reported that human osteoblasts can penetrate interconnections over 20 µm in size, and the most desirable size is over 40 µm.[22] Flautre et al. investigated the penetration of bone cells for pore interconnection with different sizes (30-130 µm), and concluded that 130 µm interconnection size led to best results for osteoconduction.[23]

The presence of small pores in the walls of the scaffolds in this study resulted in surfaces with microroughness that can enhance cell adhesion and proliferation. As mentioned earlier, the pore wall thickness increases with the increase in the amount of HA in the structure. The analysis of the pores in the pore walls for different compositions showed that the number of the pores in the walls is larger where there is HA in the structure of the $P_{DL}LA$ scaffold and where the pore walls are thicker. Similar to the average pore size and pore size distribution for the 3D structure of these scaffolds, the size of the pores in the walls and the size of pore interconnections decrease with the increase in the amount of HA in the structure. As mentioned earlier, this can be due to the increase in the viscosity of the matrix and lower diffusion rate of CO_2 into the matrix with the HA addition.

As it is presented in Table 2, all scaffolds with various compositions showed anisotropic structure similar to that of a cancellous bone.[2] The degree of anisotropy in the

3D morphology of polymer foam increases with the incorporation of HA to the structure. This increase in degree of anisotropy is due to HA reducing the diffusion rate of CO_2 into the structure, which limits efficient foam expansion and porous structure formation and causes the formation of foams with nonuniform pore structure. The $scCO_2$ processing technique has been reported to be the main reason for inducing anisotropic architecture for scaffolds, and giving rise to oriented and elongated pores in the structure.[4] Figure 7a presents a 3D image of a porous $P_{DL}LA$ scaffold created by CTVol software, and Figure 7b shows a 3D image of the surface of the same scaffold. As we previously reported, morphological parameters including porosity, pore size and pore wall thickness can be tailored by varying the processing conditions of the $scCO_2$ foaming technique.[19]

[insert Figure 7.]

Mechanical testing

The average compressive stress for the neat $P_{DL}LA$ porous scaffolds was 16.72 ± 2.99 MPa (Figure 8a). This value increased to 29.94 ± 5.07 MPa for $P_{DL}LA$ scaffold with 2 wt% HA in the structure, and 36.03 ± 5.90 MPa for 4 wt% HA. As HA has a higher compressive strength than $P_{DL}LA$ it would be predicted that HA would strengthen the pore walls of the scaffolds leading to an increase in compressive strength for the $P_{DL}LA$/HA composite prepared by $scCO_2$ foaming.[15,16] Another reason for enhanced mechanical strength obtained in this study can be the high pressure used for the fabrication of porous structures. The applied high pressure in the foaming process may result in more closely packed polymer chains.[15] The mechanical strength of the prepared scaffolds in this study are suitable for cancellous bone regeneration since 5-10 MPa has been reported as sufficient strength for cancellous bone regeneration. Further improvement in compressive strength may be obtained by adding a larger amount of HA into the structure of the scaffolds.[4,15]

[insert Figure 8.]

The mechanical behaviour of a biodegradable scaffold designed for cancellous bone regeneration needs to match that of a cellular material since cancellous bone behaves similar to a cellular material. The compressive stress-strain curve for cancellous bone or a cellular material must possess three distinct regimes which can be seen in Figure 8b. These three stages were observed for all three compositions. The curves for these porous structures start with a linear elastic regime at low stresses which is followed by a long collapse stress plateau, then by a steep increase in stress at higher strains. The linear elasticity observed in the first stage is controlled by pore wall bending for an open pore structure. The plateau is associated with collapse of pore walls, and the final sharp rise in stress occurs when the pores or cellular structure completely collapses. As a result, pore walls touch and larger strain compresses the solid material itself.[2] It can be observed in Figure 8b in the third stage that there is a large increase in modulus with the addition of HA to the structure of the pure polymer scaffold.

The compressive modulus of the scaffolds with different compositions is presented in Figure 9a. This figure shows the relationship between the porosity and pore diameter and compressive modulus of these scaffolds. The relationship between the average pore wall thickness and the compressive modulus is exhibited in Figure 9b.

[insert Figure 9.]

As it is presented in Figure 9a, the compressive modulus was measured at 17.65 ± 3.66 MPa for the porous scaffold of neat $P_{DL}LA$, and it increased to 31.61 ± 5.89 MPa for $P_{DL}LA$-2 wt% HA and 37.97 ± 6.84 MPa for $P_{DL}LA$-4 wt% HA. The increase in compressive modulus with the addition of HA to the structure can be explained by the fact that HA

strengthens the pore walls of the polymer scaffold, and reinforces the skeleton of the polymer matrix.[4,15,16,25]

A relationship between the morphological and mechanical properties has been reported in several studies.[11,16] The Gibson-Ashby model has proposed a reciprocal relationship between the pore diameter and porosity, and mechanical strength and modulus.[2] An increase in porosity and pore size can improve cell culture and metabolic activities, however, it may result in reduced compressive strength for the scaffolds.[16] Figure 9a shows that porosity and average pore diameter decrease with the addition of HA to the structure, and these possess a reciprocal relationship with the compressive modulus and according to Figure 9a with the compressive stress of the scaffolds.

It can also be seen in Figure 9b that the average pore wall thickness increases with the addition of HA to the structure, and it possesses a direct relationship with the compressive modulus. HA reinforces the skeleton of the $P_{DL}LA$ matrix leading to the formation of thicker pore walls.[4] Therefore, the composite foams with thicker pore walls tend to be more resistant to compression compared to neat $P_{DL}LA$ foams with thinner pore walls. Lower porosity and smaller pores also provide a better support for the skeleton matrix resulting in higher compressive stiffness for the composite scaffolds.

$ScCO_2$ foaming technique has been reported as a technique which produces porous scaffolds with low mechanical properties in several studies.[1,5,16] The mechanical properties obtained for porous $P_{DL}LA$ and $P_{DL}LA$/HA scaffolds in several previous studies are presented in Table 4. These values are compared with those have been measured in the current study.

Table 4. Comparison between the mechanical and morphological properties obtained for porous scaffolds in previous studies and those obtained in the current study.

Research study	Mechanical properties	Porosity
Silva et al., 2006	Compressive strength of 0.85 ± 0.1 MPa and compressive Modulus of 7.0 ± 1.7 MPa for porous $P_{DL}LA$ scaffolds, compressive strength of 1.76 ± 0.25 MPa and compressive modulus of 1.13 ± 0.26 MPa for porous $P_{DL}LA$/HA scaffolds	----
Lin et al., 2003[27]	Compressive strength of 5 MPa for porous poly(L-lactide-co-DL-lactide 70:30)	28.5%
Harris et al., 1998 (Combination of CO_2 foaming and salt leaching techniques)	Compressive modulus of 289 ± 25 kPa for porous PLGA scaffolds	95%
Barry et al., 2006	Compressive strength of 2.67 ± 0.38 MPa for porous $P_{DL}LA$ scaffolds	81.7%
Teng et al., 2007	Compressive strength of 729 kPa for porous $P_{DL}LA$ scaffold, 853 kPa for $P_{DL}LA$-2 wt% HA scaffold, 1 MPa for $P_{DL}LA$-4 wt% HA	----
Current study	Compressive strength and modulus of 16.72 ± 2.99 MPa and 17.65 ± 3.66 MPa for porous $P_{DL}LA$ scaffolds	92.39 ± 1.2% for $P_{DL}LA$ scaffolds
	Compressive strength and modulus of 29.94 ± 5.07 MPa and 31.61 ± 5.89 MPa for $P_{DL}LA$-2 wt% HA scaffolds	90.69 ± 4.3% for $P_{DL}LA$-2 wt% HA
	Compressive strength and modulus of 36.03 ± 5.90 MPa and 37.97 ± 6.84 MPa for $P_{DL}LA$-4 wt% HA scaffolds	89.47 ± 3.9% for $P_{DL}LA$-4 wt% HA

$ScCO_2$ technique provides the possibility to tailor the mechanical properties of the porous scaffolds according to the mechanical needs of the target tissue by altering the processing parameters. This is the result of the good control on porosity, pore diameter and wall thickness with $scCO_2$ technique.

Cell culture study

Templates of $P_{DL}LA$ and $P_{DL}LA$/HA composite scaffolds with three different compositions were seeded with human osteoblastic human cells to assess the ability of the foamed matrices to permit cell adhesion and growth in vitro. The resazurin assay on day 1 showed there were viable cells present on all matrices. Relative viable cell number was measured on days 1, 4 and 7 using microplate fluorescence reader.

The cells maintained viability after 7 days in culture. On day 4, matrices with various compositions contained significantly more metabolically active cells compared to day 1, as assayed by resasurin fluorescence, and there was a further increase in the number of cells between days 4 and 7, indicating the cells proliferated over the culture period (Figure 10a). The addition of HA did not affect the biocompatibility of the $P_{DL}LA$ scaffolds. There were slightly more viable cells in HA containing scaffolds, though this difference was not statistically significant. This could be because low amounts of HA were added to the structure of the polymer, and these small amounts of HA were not sufficient to improve the biocompatibility of the polymer scaffolds. Due to high biocompatibility and excellent osteoconductive properties of HA, addition of higher amount of HA to the polymer structure can potentially result in significant improvement in cell viability and biocompatibility of the $P_{DL}LA$ scaffold.[28,29] Fluorescence microscopy of fixed cells stained on day 7 confirmed there was dense cell coverage throughout the scaffolds (Figures 10b to d).

[insert Figure 10.]

Conclusions

Porous $P_{DL}LA$ scaffolds with 0, 2 and 4 wt% HA in the structure have been produced by a novel $scCO_2$ foaming technique. A comparison was made of various compositions and foaming conditions. Morphological characterisations of the scaffolds including porosity, pore diameter and pore wall thickness both on the surface and in the bulk were determined using

optical microscope and micro-CT respectively. Suitable value of pore size and structure for successful cancellous bone regeneration was obtained for all scaffolds. Pore interconnections and surface roughness were also investigated, and it was shown that suitable size of interconnections and microrough surfaces were obtained for the scaffolds in this study, which can result in enhanced cell adhesion and behaviour in the scaffolds. Compression testing was also performed on these scaffolds, and compressive strength and modulus were measured and compared for scaffolds with various compositions. Compressive strength and modulus of up to 36.03 ± 5.90 MPa and 37.97 ± 6.84 MPa were measured for the porous scaffolds with different compositions. These mechanical properties showed an improvement with the addition of HA to the structure. Also, the relationship between morphological and mechanical properties was investigated. Compressive strength and modulus increased as the pore wall thickness increased or porosity and pore diameter decreased. As a result of the good control on the morphology of the porous scaffolds in this $scCO_2$ foaming technique, the mechanical properties of the scaffolds can be tailored to the mechanical requirements of the target tissue by altering the porosity, pore diameter or pore wall thickness. All scaffolds with different compositions presented a high cell viability and biocompatibility following bone cells seeding on the matrices. The maintenance of good cell viability demonstrates that there was no toxicity of the material or the processing parameters. The number of cells attached on the matrices slightly improved with the addition of HA, however, there was no significant improvement in biocompatibility of the scaffolds. A future study can involve a cell culture study on the bulk of the scaffolds rather than a thin section of the structure. This can provide the possibility to study the cell infiltration and investigate the pore interconnection further. The results in this study showed that fully interconnected porous biodegradable scaffolds with enhanced mechanical strength, desirable morphology and biocompatibility can be achieved using $scCO_2$ foaming.

Acknowledgements

We wish to thank The Mellanby Centre for bone research for their technical support with their micro-CT unit.

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