European Cells and Materials Vol. 14. 2007 (pages 64-77) DOI: 10.22203/eCM.v014a07 ISSN 1473-2262

CONTROL OF PORE SIZE AND STRUCTURE OF TISSUE ENGINEERING SCAFFOLDS PRODUCED BY SUPERCRITICAL FLUID PROCESSING

Hongyun Tai[1,2], Melissa L. Mather[3], Daniel Howard[2], Wenxin Wang[1], Lisa J. White[2], John A. Crowe[3], Steve P. Morgan[3], Amit Chandra[4], David J. Williams[4], Steven M. Howdle[1*] and Kevin M. Shakesheff[2]

[1] School of Chemistry, [2] School of Pharmacy, [3] School of Electrical and Electronic Engineering, The University of Nottingham, University Park, Nottingham, NG7 2RD
[4] School of Mechanical and Manufacturing Engineering, The University of Loughborough, Loughborough, Leicestershire, UK, LE11 3TU

Abstract

Tissue engineering scaffolds require a controlled pore size and structure to host tissue formation. Supercritical carbon dioxide ($scCO_2$) processing may be used to form foamed scaffolds in which the escape of CO_2 from a plasticized polymer melt generates gas bubbles that shape the developing pores. The process of forming these scaffolds involves a simultaneous change in phase in the CO_2 and the polymer, resulting in rapid expansion of a surface area and changes in polymer rheological properties. Hence, the process is difficult to control with respect to the desired final pore size and structure. In this paper, we describe a detailed study of the effect of polymer chemical composition, molecular weight and processing parameters on final scaffold characteristics. The study focuses on poly(DL-lactic acid) ($P_{DL}LA$) and poly(DL-lactic acid-co-glycolic acid) (PLGA) as polymer classes with potential application as controlled release scaffolds for growth factor delivery. Processing parameters under investigation were temperature (from 5 to 55°C) and pressure (from 60 to 230 bar). A series of amorphous $P_{DL}LA$ and PLGA polymers with various molecular weights (from 13 KD to 96 KD) and/or chemical compositions (the mole percentage of glycolic acid in the polymers was 0, 15, 25, 35 and 50 respectively) were employed. The resulting scaffolds were characterised by optical microscopy, scanning electron microscopy (SEM), and micro X-ray computed tomography (μCT). This is the first detailed study on using these series polymers for scaffold formation by supercritical technique. This study has demonstrated that the pore size and structure of the supercritical $P_{DL}LA$ and PLGA scaffolds can be tailored by careful control of processing conditions.

Key Words: poly(DL-lactic acid) ($P_{DL}LA$), poly(lactic acid-co-glycolic acid) (PLGA), supercritical carbon dioxide ($scCO_2$), plasticization, foaming, scaffolds

*Address for correspondence:
Steven M. Howdle
School of Chemistry
The University of Nottingham
University Park
Nottingham, NG 7 2RD, UK
Email: steve.howdle@nottingham.ac.uk

Introduction

In tissue engineering, a porous scaffold is required to act as a template and guide for cell proliferation, differentiation and tissue growth. Scaffolds may also act as controlled release devices that deliver growth factors with rates matching the physiological need of the regenerating tissue (Langer, 1998). Poly(lactic acid) (PLA) and associated poly(lactic acid-co-glycolic acid) (PLGA) copolymers are commonly used biodegradable polymers for fabricating tissue engineering porous scaffolds. PLGA copolymers with various polymer compositions (the ratio of lactic acid and glycolic acid content in the polymer) degrade at different rates. Therefore, it is of great interest using PLGA copolymers to make scaffolds for various applications. These polymers degrade *in vivo* and eventually disappear at a desired rate while the native tissues grow and the degradation residues are discharged through rental filtration. Moreover, the release of encapsulated growth factors from these materials depends on both diffusion and degradation.

Common scaffold fabrication techniques include solvent casting/salt leaching (Lu *et al*., 2000; Murphy *et al*., 2002), moulding/salt leaching (Hou *et al*., 2003; Sosnowski *et al*., 2006) and gas foaming agent/salt leaching (Kim *et al*., 2006; Nam *et al*., 2000). These conventional methods require the use of organic solvents and/or elevated processing temperatures which can prohibit their use in the preparation of growth factor loaded scaffolds. Additionally, the salt leaching process may lead to the removal of a proportion of the growth factor dose during prolonged leaching (Hutmacher, 2000). To overcome these limitations, carbon dioxide (CO_2) has been used as a plasticiser and foaming agent to form three-dimensional (3-D) scaffolds (Barry *et al*., 2004; Barry *et al*., 2006; Harris *et al*., 1998; Hile *et al*., 2000; Hile and Pishko, 2004; Howdle *et al.*, 2001; Mooney *et al*., 1996; Quirk *et al*., 2004; Singh *et al*., 2004; Watson *et al*., 2002; Yang *et al*., 2004). CO_2 is inexpensive, non-toxic and non-flammable and readily available in high purity from a variety of sources. Supercritical carbon dioxide, $scCO_2$, (T_c = 31.1°C, P_c = 73.8 bar) has the combination of gas-like diffusivity and liquid-like density, which makes $scCO_2$ a unique medium for polymer synthesis and polymer-processing (Cooper 2001; Tomasko *et al*., 2003; Woods *et al*., 2004). The addition of small amounts of compressed CO_2 to polymer phases can result in substantial and

Table 1: $P_{DL}LA$ and PLGA polymer characteristics.

Polymers	Resource	Form	Composition (L:G)	Inherent viscosity (dL/g)	M_w[a] (KD)	PDI[b]	$T_{g,DSC}$[c] (^oC)
$P_{DL}LA$(52K)	Purac	Granular	100:0	0.52	52	1.87	46.9
PLGA 85:15(15K)	Lakeshore	Pellet	85:15	0.18	15	1.76	40.2
PLGA 85:15(39K)	Lakeshore	Pellet	85:15	0.41	39	1.81	49.2
PLGA 85:15(77K)	Lakeshore	Pellet	85:15	0.63	77	1.70	48.6
PLGA 85:15(96K)	Lakeshore	Pellet	85:15	0.73	96	1.70	49.5
PLGA 75:25(13K)	Resomer	Fine powder	75:25	0.16-0.24	13	1.89	37.6
PLGA 75:25(72K)	Resomer	Fine powder	75:25	0.5-0.7	72	1.75	50.4
PLGA 65:35(52K)	Lakeshore	Granular	65:35	0.5	52	1.69	49.1
PLGA 65:35(82K)	Lakeshore	Granular	65:35	0.66	82	1.69	43.6
PLGA 50:50(53K)	Resomer	Fine powder	50:50	0.55	53	1.59	47.0

[a.]Weight average molecular weight, determined by GPC; [b.]Polydispersity, determined by GPC; [c.]Glass transition temperature, determined by DSC.

dramatic changes in the physical properties, including viscosity, permeability, interfacial tension and glass transition temperature (T_g). Mooney *et al.* (1996) formed PLGA scaffolds (the mole ratio of lactic acid and glycolic acid (L/G) was 50:50) by a CO_2 pressure quenching method. The preformed PLGA polymer discs were saturated in CO_2 at a pressure of 55 bar and ambient temperature (20-23^{o}C) for a prolonged time (48 to 72 hours). This was followed by a rapid depressurisation (*e.g.* seconds). The foams produced had pores of approximately 100 μm in diameter and porosities up to 93%, but had low interconnectivity. It was further reported that the combination of CO_2 gas foaming and salt leaching technique (GF/SL) led to scaffolds with open pore structures (Harris *et al.*, 1998; Riddle and Mooney, 2004). Subsequently, PLGA copolymers with various compositions, L/G ratios of 85:15, 75:25, 65:35 and 50:50, were employed to fabricate porous scaffolds by the GF/SL technique for the controlled delivery of vascular endothelial growth factor (VEGF) (Murphy *et al*., 2000; Sheridan *et al*., 2000), DNA (Jang and Shea, 2003) and as a support for 3-D culture of cells (Quirk *et al*., 2004). Pishko and co-workers (Hile *et al*., 2000; Hile and Pishko, 2004) also produced PLGA (L/G ratio as 80:20 and 65:35) scaffolds using water-in-solvent emulsion (aqueous protein phase and organic polymer solution phase) *via* a CO_2 pressure quenching method at conditions in the supercritical region (35^{o}C, 80 bar) for a prolonged saturation time (24 hours) to deliver basic fibroblast growth factor (bFGF).

By contrast, we have developed a single step supercritical carbon dioxide ($scCO_2$) foaming process using polymer powder samples to generate porous scaffolds (Howdle *et al.*, 2001). This novel $scCO_2$ foaming process has been carried out at high pressure (170-230 bar) and short soaking times (0.5-2 hours) with a controlled venting rate (venting time between 2 minutes and 2 hours) at 35^{o}C (Barry *et al*., 2004; Barry *et al*., 2006; Howdle *et al*., 2001; Quirk *et al*., 2004; Watson *et al*., 2002; Yang *et al*., 2004). The produced scaffolds with an interconnected porous structure (pore size *ca.* 200-500 μm and porosity *ca.* 60 - 80 %) have been studied for growth factor and gene delivery. For example, bone morphogenetic protein 2 (BMP-2) has been encapsulated into $P_{DL}LA$ scaffolds for bone tissue engineering by this supercritical fluid mixing and foaming technique (Yang *et al*., 2004). Bone formation was observed due to the release of the osteoinductive protein BMP-2 from $P_{DL}LA$ scaffolds both *in vitro* and *in-vivo* (Yang *et al*., 2004; Yang *et al.*, 2006). These scaffolds have also been used to study adenoviral gene transfer into primary human bone marrow osteoprogenitor cells (Partridge *et al*., 2002; Howard *et al*., 2002). Recently, polyamidoamine (PAA)/DNA complexes have been incorporated into supercritical $P_{DL}LA$ scaffolds; these exhibited a slow release and extended gene expression profile (Heyde *et al*., 2007).

Scaffolds with a desired pore size, porosity and interconnectivity are required by various tissue engineering applications because the pore structure strongly influences cell growth and drug release profile. However, the process of forming scaffolds by supercritical fluid technique is difficult to control with respect to changes in final pore size and structure. In this paper, a series of poly(DL-lactic acid) ($P_{DL}LA$) and poly(DL-lactic acid-co-glycolic acid) (PLGA) polymers were employed for a detailed study of the effect of polymer composition, molecular weight and processing parameters on final scaffold characteristics.

Experimental

Materials

In this study, a series of amorphous $P_{DL}LA$ and PLGA polymers with different inherent viscosity and compositions were purchased from Boehringer Ingelheim (Resomer, Germany), Purac (Gorinchem, the Netherlands) and Lakeshore Biomaterials (Birmingham, AL, USA) in the forms of fine powder, granular or pellet, and used as received (Table 1). Weight-average molecular weights (M_w) and the polydispersity (PDI) of these polymers were measured by Gel Permeation Chromatography (GPC) (PL-120, Polymer Laboratories Ltd., Church Stretton, Shropshire, UK) with a refractive index (RI) detector. The columns (30 cm PLgel Mixed-C, two in series) were eluted by tetrahydrofuran (THF) and calibrated with narrow molecular weight distribution polystyrene standards. All calibrations and analyses were performed at 40^{o}C with a

flow rate of 1 ml/min. The glass transition temperatures (T_g) of these polymers were determined utilising a TA 2920 Differential Scanning Calorimeter (DSC). The tests were performed from -10 to 120°C, at a heating rate of 10°C/min. Food grade CO_2 was supplied by Cryoservice and used without further purification.

Scaffolds production by CO_2 foaming technique

130 mg of polymer was weighed into each well of a Teflon mould (12 wells with 10 mm diameter and 10 mm height, no top-lid, made in house) as described previously (Quirk *et al*., 2004). This mould was designed with a detachable base to allow easy removal of scaffolds after fabrication. The mould was then placed into a 60 mL clamp sealed stainless steel high-pressure autoclave (made in house) (Furno *et al*., 2003). The autoclave was equipped with a pressure transducer for pressure monitoring and a heating jacket with a CAL 3300 temperature controller for temperature control. HIP high pressure valves and Swagelok tubing and fittings were adopted to connect the system. A high pressure PM101 pump (New Ways of Analytics, Lörrach (Baden-Württemberg), Germany) was used to charge CO_2 into the autoclave. The vessel was heated or cooled to a desired temperature (T), and then pressurised to a certain pressure (P) over a period of filling time (FT). After the polymer/CO_2 mixture was maintained at the constant pressure P for a desired soaking time (ST), the vessel was then depressurised to ambient pressure over a period of venting time (VT). The three stages of the scaffold production process, *i.e.* filling, soaking and venting, were controlled by a backpressure regulator (BPR, Bronkhorst, the Netherlands) *via* a computer program. Porous scaffolds with a layer of nonporous skin and the size of approximately 10 mm diameter and 5-10 mm height were generated by this procedure. It is worth to mention that for cell seeding and drug release studies, the nonporous skin of the scaffolds should be removed by cutting with a scalpel blade.

Characterisation of scaffolds

Microscope optical images of scaffolds were captured using a Cool Snap Pro digital camera equipped with a Nikon (Kingston-upon-Thames, U.K.) AF Micro Nikkor 60 mm f/2.8D lens and interfaced through a CoolSnap Pro PCI interface card (MDS, Toronto, Canada) to a Pentium PIII 1Ghz PC. The images were acquired and processed using image analysis software (Image Pro Plus 4.5, Media Cybernetics, Bethesda, MD, USA).

Scanning Electron Microscopy (SEM) was used to determine the foam morphology of scaffolds at the cross section. A scalpel blade was used to cut the scaffold in half vertically. The sample was mounted on an aluminium stub using an adhesive carbon tab and sputter coated with gold before images were obtained using a JEOL (Tokyo, Japan) JSM-6060LV SEM machine.

Micro X-ray Computed Tomography (Micro-CT) images were obtained using a high resolution micro-CT system (μCT 40, Scanco Medical, Bassersdorf, Switzerland). Scaffolds were mounted on a rotary stage inside the micro-CT apparatus and scanned. The scanner was set to a voltage of 55 kV and a current of 143 mA. The resulting 2-D images were used to construct 3-D images.

Results and Discussion

Visual observation of plasticisation and foaming process

A double headed clamp sealed view cell (100 ml) with a sapphire widow was used to carry out visual observations. The images were captured by a Thorlabs (Newton, NJ, USA) CCD (couple charged device) camera. A small glass vial (10 mm diameter and 10 mm height) was used as the container for the polymer sample. The production of a scaffold was carried out as described in the experimental section. When the CO_2 was charged into the vessel, a decrease of the apparent volume of the solid sample was observed, indicating that the polymer sample was plasticised into a liquid-like state, which was denser than a loose packed powder or pellet sample. This plasticsation took place rapidly (within minutes) depending on the soaking pressure employed. It occurred immediately after the pressure reached 230 bar, and within 10 minutes for a lower pressure of 50 bar. The plasticised polymer was a transparent liquefied polymer melt (Figure 1a). Upon depressurisation, the transparent swollen polymer became opaque from the top surface to the bottom of the polymer/CO_2 mixture (Figure1b), indicating phase separation had occurred in the polymer/CO_2 mixture. A liquid and gas boundary in the sample container was clearly observed (Figure 1c) during rapid depressurisation because the

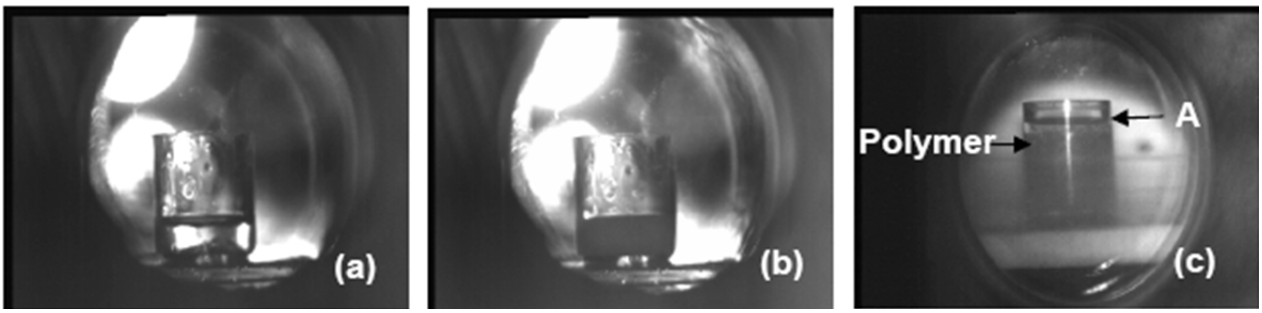

Figure 1: View cell observations: (a) plasticised polymers appeared to be transparent liquefied polymer melts; (b) plasticised polymers became opaque while venting occurred, indicating phase separation and gas nucleation; (c) phase separation observed in CO_2 fluid phase while fast venting was operated. A liquid/gas boundary (A) in the sample container was clearly observed.

Table 2: Pore diameter (μm) and porosity (%) of scaffolds fabricated at T = 35°C, P = 230 bar, FT = 20 minutes, ST = 60 minutes, VT = 60 minutes.

		Pore Diameter [a]		
Entry	Polymers	Mean (μm)	SD[b] (μm)	Porosity % [c]
1	P_{DL}LA(52K)	580.1	154.0	78
2	PLGA 85:15(39K)	323.9	101.3	-
3	PLGA 85:15(77K)	199.4	46.0	70
4	PLGA 85:15(96K)	81.9	25.4	-
5	PLGA 75:25(72K)	70.6	16.4	72
6	PLGA 65:35(52K)	65.1	11.1	78
7	PLGA 65:35(81K)	49.4	11.8	-
8	PLGA 50:50 (53K)	38.8	8.7	34

[a] Determined by SEM; [b] Standard deviation; [c] Determined by Micro CT, standard deviation ~2%.

temperature in the vessel dropped dramatically leading to a phase separation in the CO_2 fluid phase. The expansion of opaque polymers into foams was observed while venting continued.

Effect of molecular weight and chemical composition of P_{DL}LA and PLGA polymers

To study the effect of molecular weight and the composition of the polymers, scaffolds were fabricated using a series of P_{DL}LA and PLGA polymers (Table 1) under the same fabrication conditions at T = 35°C, P = 230 bar, FT = 20 minutes, ST = 60 minutes and VT = 60 minutes. The results demonstrate that the pore size of the scaffolds produced decreases with increasing molecular weight (Table 2, entries 2-4 for PLGA 85:15 and entries 6-7 for PLGA 65:35). Moreover, the pore size decreases with increasing glycolic acid content (Figure 2 and Table 2, entries 1, 3, 5, 6, 8). The SEM images of the P_{DL}LA scaffolds showed more open and interconnected pore structures than for the PLGA scaffolds (Figure 2). The pore size distribution of the porous scaffolds, determined by micro CT, is much narrower for those produced from PLGA copolymers than those from P_{DL}LA homopolymer. There is also a trend in the pore size distribution which becomes narrower with increasing glycolic acid content of the copolymers (Figure 3a). The porosity of P_{DL}LA and PLGA scaffolds, determined by micro CT, indicated that the scaffolds possess porosities as high as 78%; the scaffolds produced from PLGA with high glycolic acid content had a lower porosity (Table 2, entry 8). In addition, the scaffolds generated from polymers with low molecular weight, *e.g.* PLGA 85:15(15 KD) and PLGA 75:25(13 KD) appeared to be the most fragile of the whole set.

The critical parameters for controlling foam development in a CO_2 foaming process are the concentration of CO_2 in the polymer and the rate of CO_2 escaping from the polymer (Stafford *et al*., 1999). These are closely related to the solubility of CO_2 in the polymers, which is dependent upon the molecular structure and morphology of the polymers. Recent studies have demonstrated that CO_2 has much lower solubility in semi-crystalline polymers than in amorphous polymers due to the free volume effect (Shieh *et al*., 1996a, Shieh *et al*., 1996b, Oliverira *et al*., 2006b). Oliveira *et al*. (2006a,

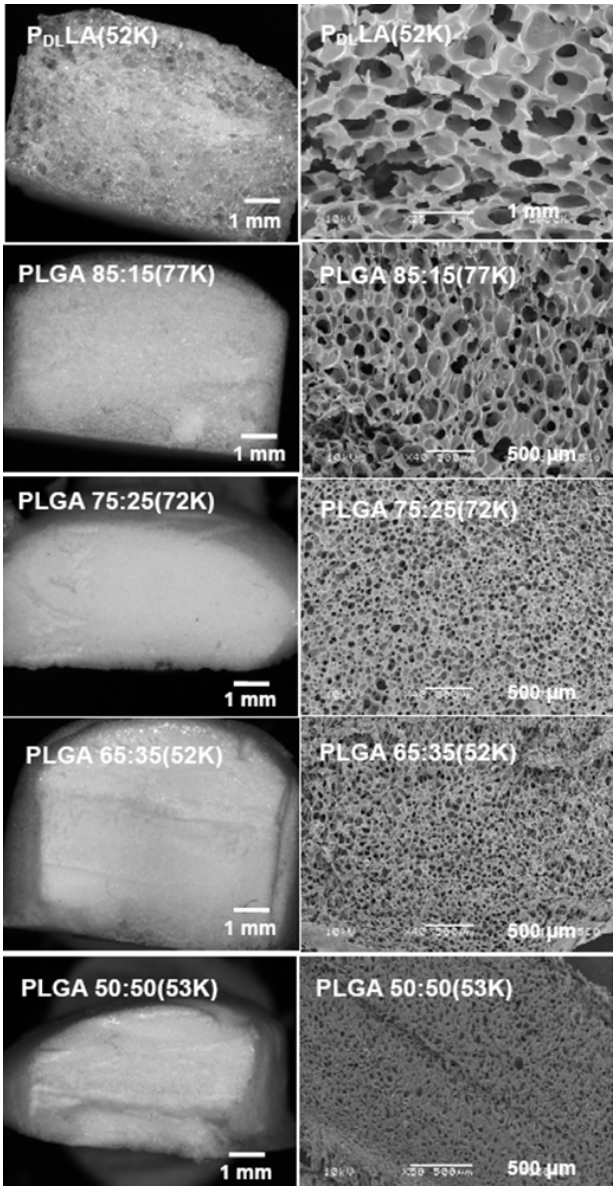

Figure 2: The effect of polymer composition on the morphology of P_{DL}LA and PLGA scaffolds: Optical microscopy images are in the left column and SEM images are in the right column. Processing conditions: T = 35°C, P = 230 bar, FT = 20 minutes, ST = 60 minutes, VT = 60 minutes. Note, the pore size decreases with increasing glycolic acid content from *ca.* 580 to 40 μm (Table 3, entries 1, 3, 5, 6, 8).

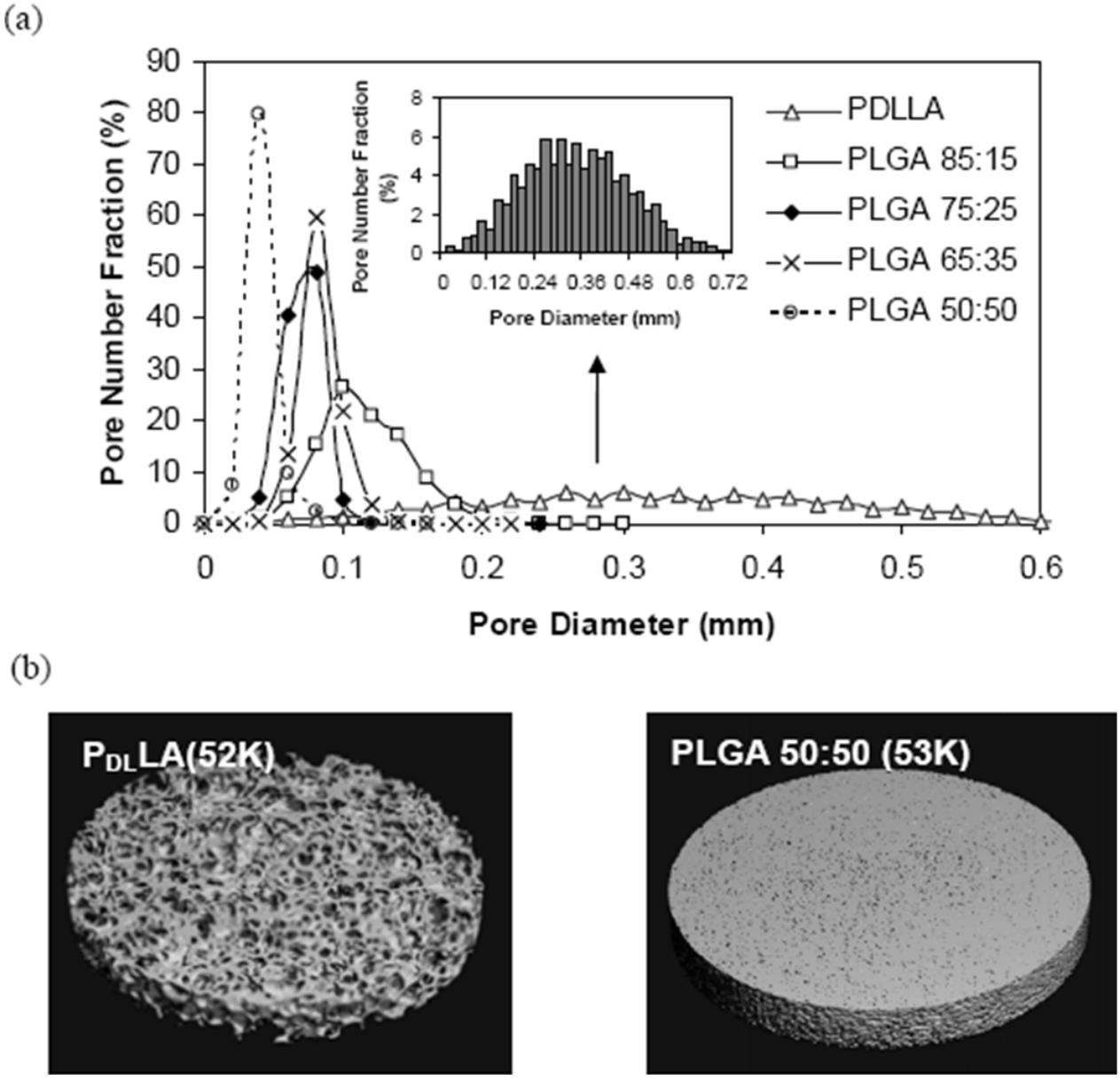

Figure 3: Scaffolds fabricated with $P_{DL}LA$(52K), PLGA 85:15(77K), PLGA 75:25(72K), PLGA 65:35(52K), and PLGA 50:50(53K) polymers with (a) pore size distribution determined by Micro CT and (b) Micro CT 3-D images. Processing conditions: T = 35°C, P = 230 bar, FT = 20 minutes, ST = 60 minutes, VT = 60 minutes.

2006b) found that the L:D ratio played a dominant role in CO_2 sorption in PLA and their results indicated that CO_2 was more soluble in PLA 80:20 (amorphous) than in PLA 98:2 (semi-crystalline). Therefore, CO_2 should have lower solubility in crystalline poly(L-lactic acid) (PLLA) than in the amorphous $P_{DL}LA$ (L:D ratio 50:50) used in this study. Nalawade *et al.* (2006) reported that polymers with ether groups displayed stronger interactions with CO_2 than polyesters. The affinity of CO_2 with polyesters is largely due to the interaction of CO_2 molecules with the carbonyl group on the polymer chains (Kazarian *et al*., 1996, Liu *et al*., 2007). It was found that the solubility of CO_2 in PLGA copolymers decreased with the increase in the glycolic acid content (Liu *et al*., 2007). To compare LA and GA molecular structures, it shows that LA possesses an extra methyl group which could lead to at least two-opposing consequences. One is to increase the steric hindrance and then lower the interaction between the carbonyl group and the CO_2 molecules, whereas the other one is to increase the available free volume in the matrix due to the steric effect. It was hypothesized that the latter factor plays a dominant role in determining the CO_2 behaviour in PLGA (Liu *et al*., 2007), leading to a higher solubility for PLGA with a high LA content. Moreover, as a result of the viscosity dependence, the molecular weight and polydispersity of the polymer should influence the structure of the foam developed. During the polymer expansions followed by the depressurization, the long chains (high molecular weight) entangle to lock CO_2 in; whereas the short chains (low molecular weight) allow easier escape of CO_2 from the polymer, therefore promote rapid pore growth leading to larger pores and the fragile structure.

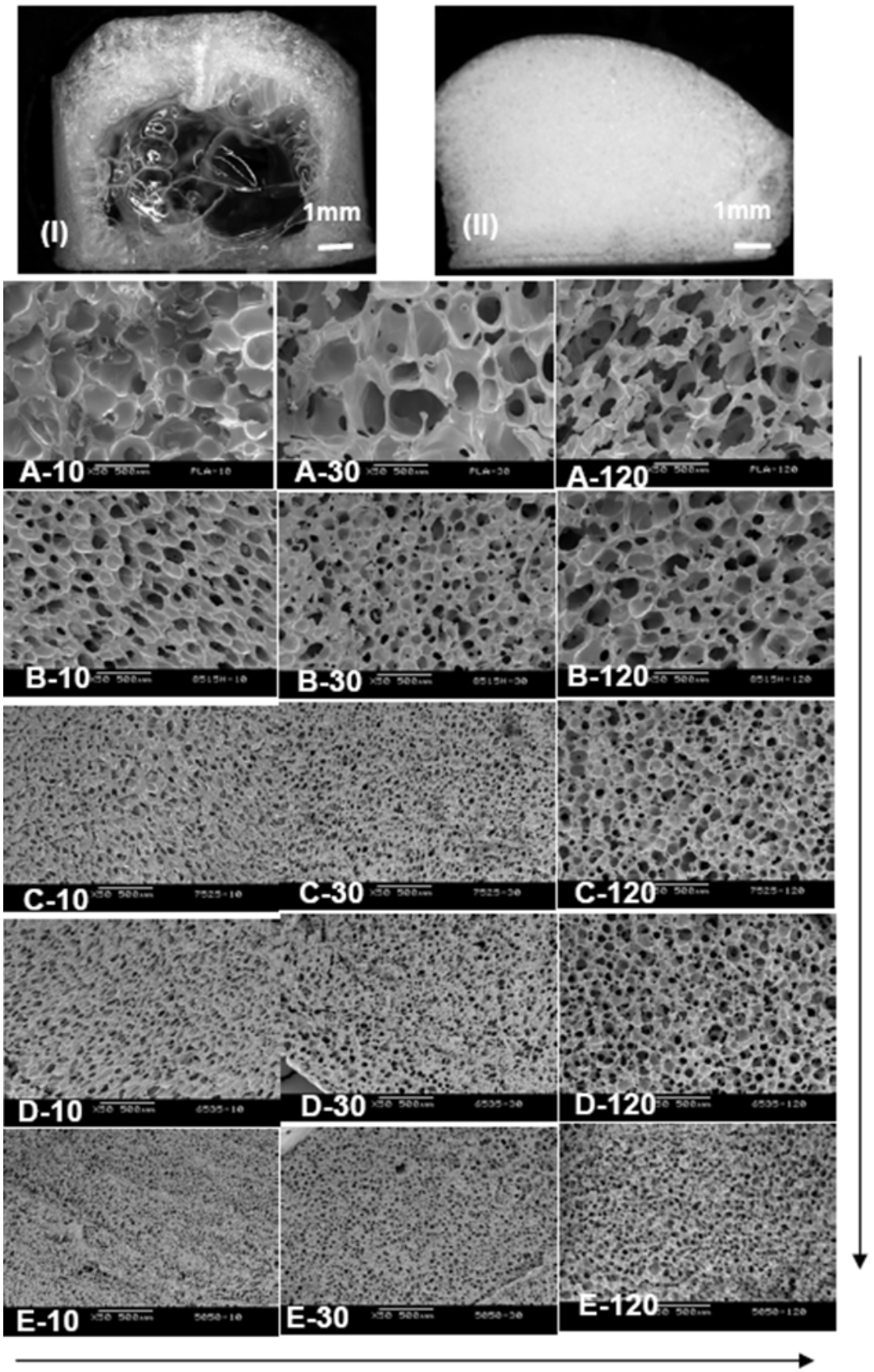

Figure 4: SEM and optical microscopy images for $P_{DL}LA$ and PLGA scaffolds fabricated at different venting rate (VT = 10, 30, 120 minutes). Other processing conditions: T = 35°C, P = 230 bar, FT = 20 minutes, ST = 60 minutes. A: $P_{DL}LA$(52K); B: PLGA 85:15(77K); C: PLGA 75:25(72K); D: PLGA 65:35(52K); E: PLGA 50:50(53K). Note, at fast venting (2 minutes), non-uniform large pores were generated (I); at slow venting (120 minutes), uniform and more open pores were generated (II). In addition, the pore size decreases from A to E (indicating by the vertical arrow), and increases with venting time from 10 to 120 minutes (indicating by the horizontal arrow). Scale bar for all SEM images is 500 μm.

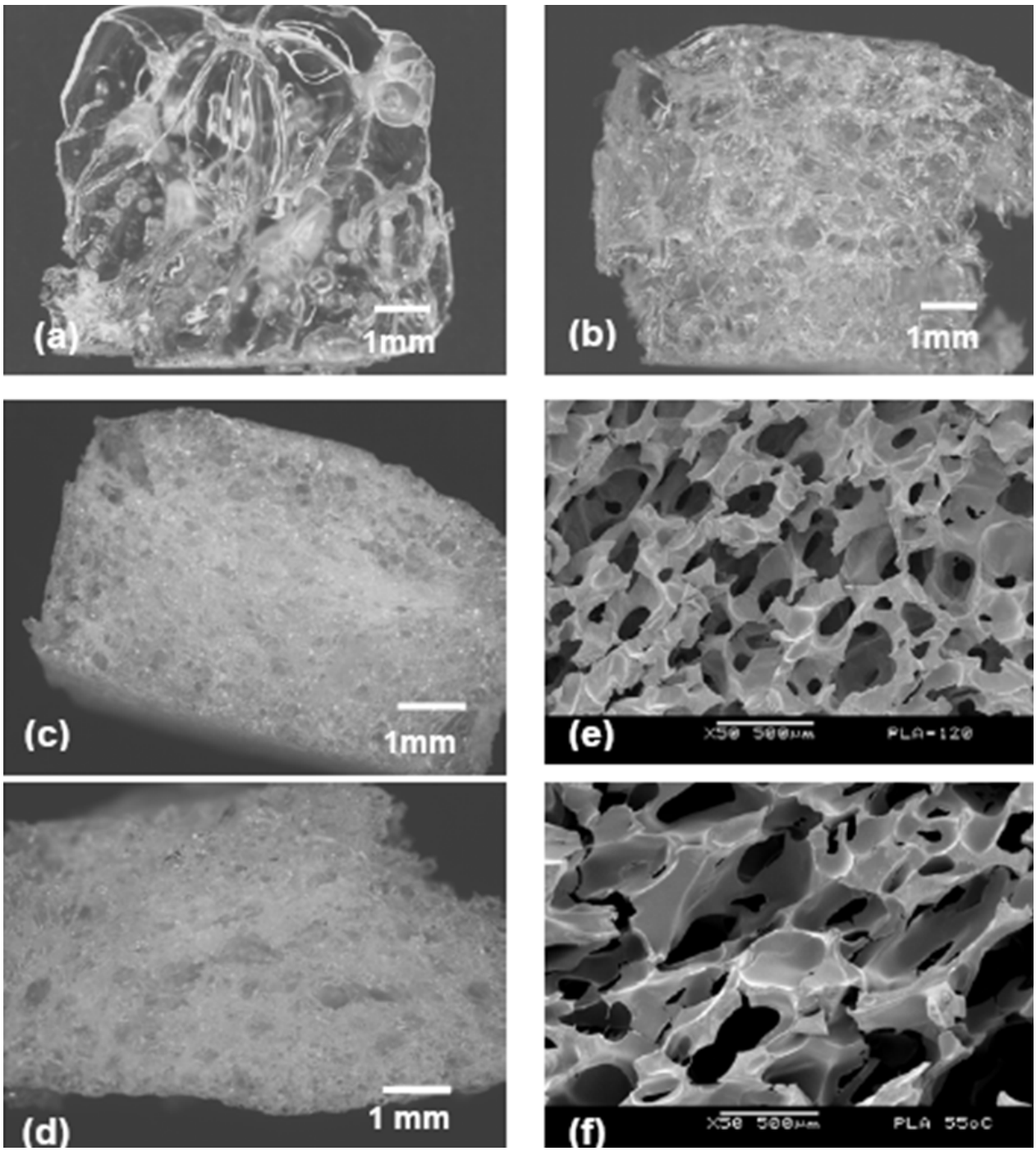

Figure 5: Images obtained by optical microscopy (a-d) and SEM (e,f) for $P_{DL}LA(52K)$ scaffolds fabricated at different temperatures with (a) 5 °C; (b) 25 °C; (c,e) 35 °C ; (d,f) 55 °C. Processing conditions: P = 230 bar, FT = 20 minutes, ST = 60 minutes, VT = 120 minutes. Scale bar for SEM images is 500 μm.

Effect of processing conditions on the morphology of $P_{DL}LA$ and PLGA scaffolds

It is well known that the solubility of CO_2 in polymers, the viscosity of swollen polymer, the nucleation density and the rate at which the CO_2 diffuses from the matrix to the growing pores are all dependent upon the processing conditions, *e.g.* temperature, pressure, pressure drop, and depressurisation rate (Arora *et al.*, 1998; Goel and Beckman, 1994a, 1994b; Sproule *et al*., 2004; Stafford *et al.*, 1999). Therefore, the resulting foam structures can be controlled by manipulating these processing conditions. In this work, five polymer samples with similar molecular weight *ca.* 50 – 80 KD but different compositions, *i.e.* $P_{DL}LA(52K)$, PLGA 85:15(77K), PLGA 75:25(72K), PLGA 65:35(52K), and PLGA 50:50(53K), were employed to study the effect of these parameters on the foam structures.

Effect of depressurisation rate. A series of experiments were carried out in which the rate of depressurisation was varied while holding the soaking conditions constant at 35°C and 230 bar for 1 hour. When depressurisation occurred rapidly at 2 and 5 minutes, the scaffolds typically displayed a non-uniform structure (Figure 4I) caused by temperature drop and phase

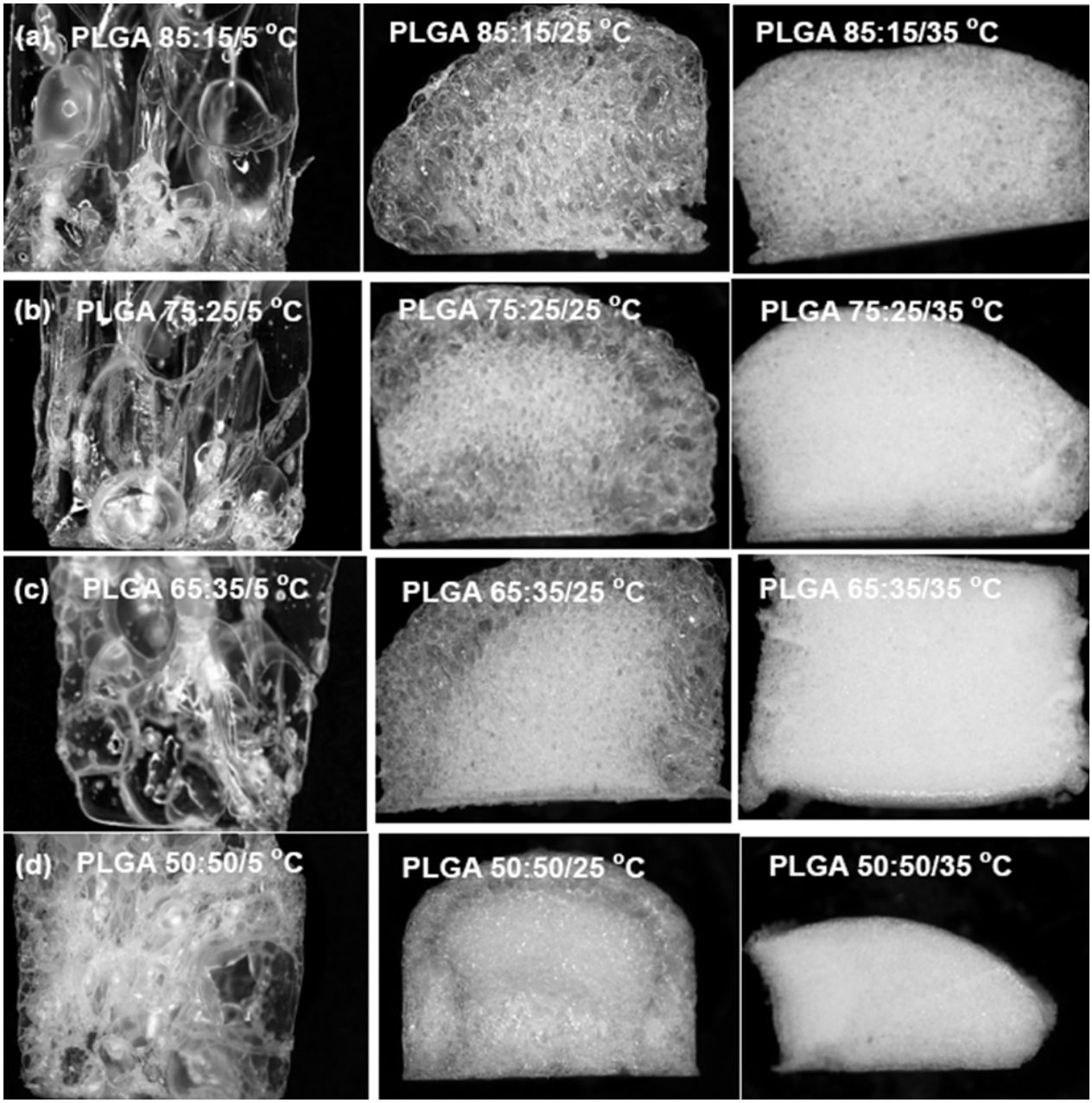

Figure 6: Optical microscopy images for PLGA scaffolds prepared at 5, 25, and 35°C. (a) PLGA 85:15(77K); (b) PLGA 75:25(72K); (c) PLGA 65:35(52K); (d) PLGA 50:50(53K). Fabrication conditions: P = 230 bar, FT = 20 minutes, ST = 60 minutes, VT = 120 minutes. The diameter of the scaffolds is *ca.* 10 mm.

separation of the system as discussed in the visual observation section. This contrasts with the uniform porous structures obtained by the well controlled slow venting process (Figure 4II). The rate of CO_2 release and the polymer relaxation are interdependent. A greater pressure drop rate resulted in higher supersaturation, leading to a greater nucleation rate. Decreasing the venting rate to 120 minutes permits the nucleation sites to grow into larger pores, whilst also allowing the pores to coalesce into more open structures (Figure 4, A-120, B-120, C-120, D-120, E-120) than those where depressurisation was more rapid, *i.e.* 10 minutes (Figure 4, A-10, B-10, C-10, D-10, E-10) and 30 minutes (Figure 4, A-30, B-30, C-30, D-30, E-30). These data agree with the published PMMA/CO_2 (Goel and Beckman, 1994a, 1994b; Sproule *et al*., 2004) and PS/CO_2 systems (Arora *et al*., 1998).

Effect of soaking temperature. Experiments varying the foaming temperature, while holding other variables constant, show that the temperatures below supercritical point (5 and 25°C) produce scaffolds with large and non-uniform pores from both $P_{DL}LA$ homopolymers (Figure 5) and PLGA copolymers (Figure 6). At the temperatures above the supercritical point (35 and 55 °C), uniform pores were formed. In addition, a higher temperature (55°C) led to larger and more open pores than a lower temperature (35°C) (Figure 7), which is in agreement with the PS/CO_2 system (Arora *et al*., 1998). The solubility of CO_2 in the polymers increases with decreasing temperature at a constant pressure because of the increase of CO_2 density. However, the diffusion rate of CO_2 in the polymers is low at a low temperature. Therefore, it is hypothesised that CO_2 might not be distributed uniformly throughout the

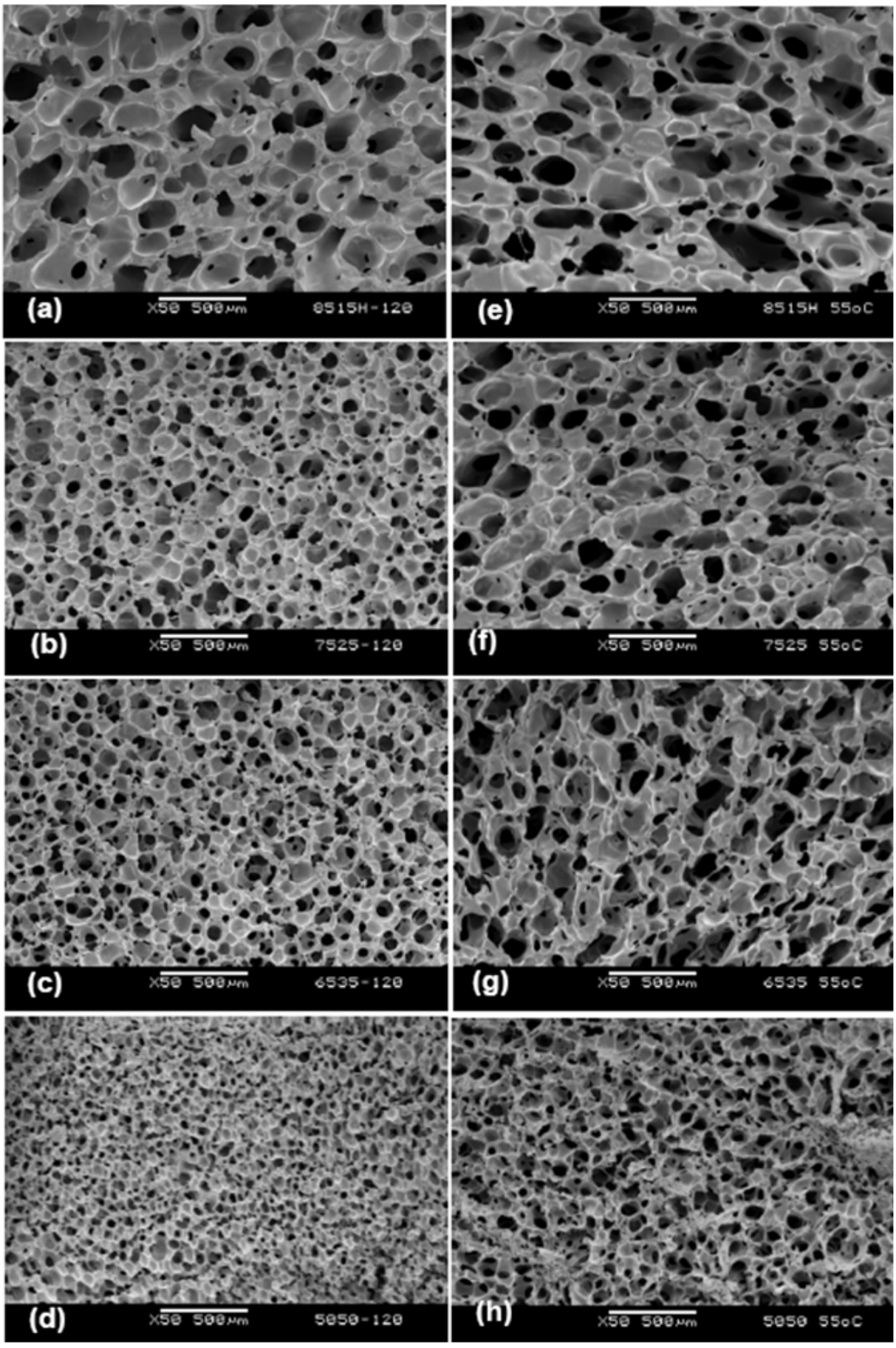

Figure 7: SEM images for PLGA scaffolds produced at 35°C (a-d) and 55°C (e-h) (a,e): PLGA 85:15(77K); (b,f): PLGA 75:25(72K); (c,g): PLGA 65:35(52K); (d,h) PLGA 50:50(53K). Note, larger pores were formed at 55°C than those at 35°C. Scale bar for all SEM images is 500 µm.

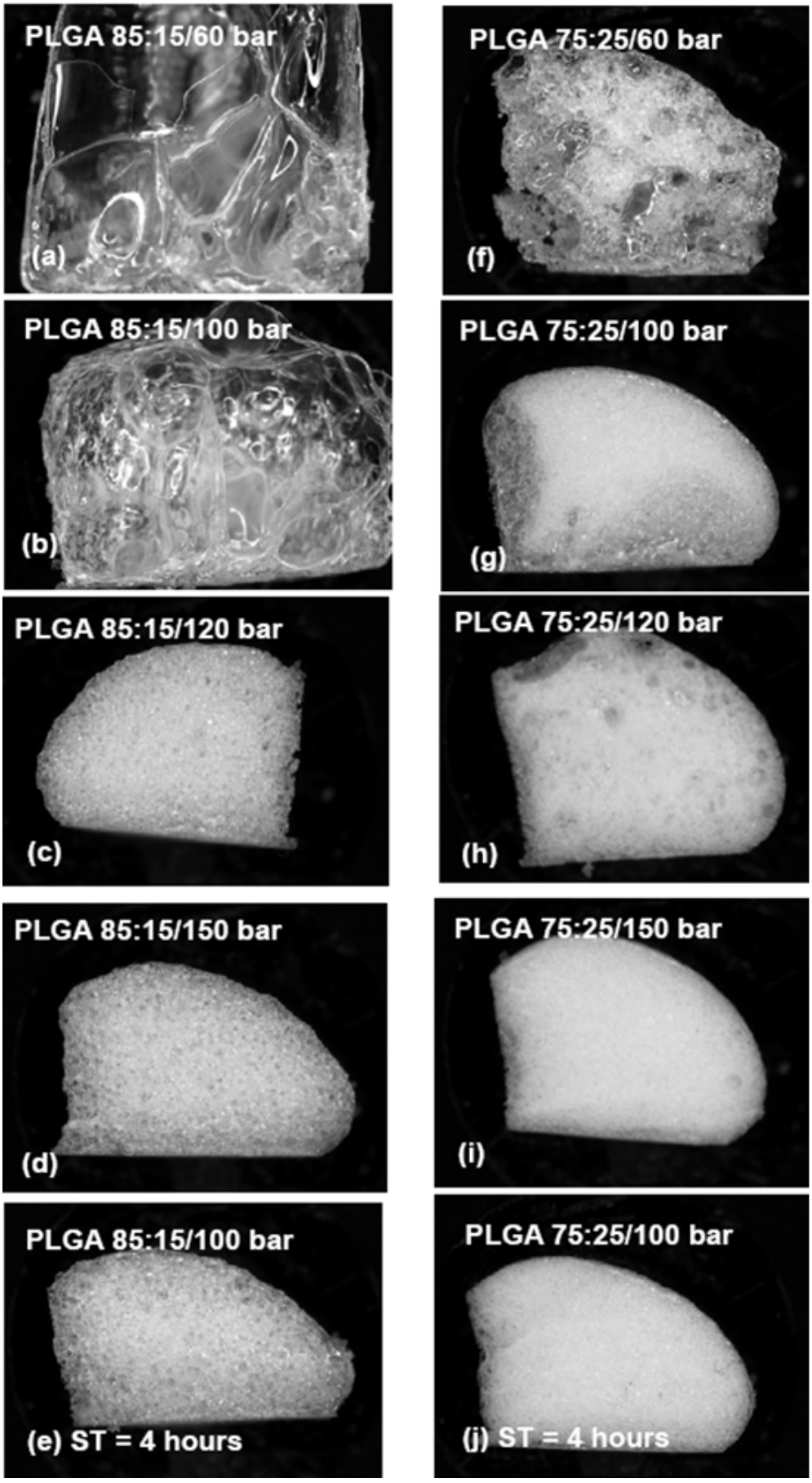

Figure 8: Optical microscopy images for PLGA 85:15(77K) (a-e) and PLGA 75:25 (72K) (f-j) scaffolds fabricated at different pressures (P) with (a,f): 60 bar; (b,g): 100 bar; (c,h): 120 bar; (d,i): 150 bar. Processing conditions: T = 35°C, FT = 20 minutes, ST = 60 minutes, VT = 120 minutes. (e,j): P = 100 bar, ST= 4 hours, T = 35°C, FT = 20 minutes, VT = 120 minutes.

swollen polymers within the soaking time (1 hour) at low temperatures. A CO_2 concentration gradient might exist, *i.e.* high CO_2 concentration regions close to the interface with CO_2. Whilst venting occurs, some regions may have been supersaturated earlier than others, leading to non-uniform nucleation sites. Also, at low temperatures, phase separation of CO_2 occurs while venting. The combination of these effects may have led to non-uniform pores at low temperatures. Moreover, this also confirms that CO_2 can depress the T_g of these biomaterials to well below body temperature (5°C). It is known that the diffusion coefficient of the swollen polymer increases with temperature, thus the time required to reach equilibrium decreases with temperature (Royer *et al*., 1999). Under high temperatures, the CO_2 was uniformly distributed in the swollen polymers and there was no phase separation while venting. In addition, a higher diffusion rate of CO_2 at 55°C allowed the pores to grow larger than those at 35°C, as can be seen in Figure 7.

Effect of soaking pressure. The effect of the soaking pressure (60, 100, 120 and 150 bar) on the foam structure was studied at a constant temperature of 35°C and the same conditions for filling, soaking and venting (FT = 20 minutes, ST = 60 minutes, VT = 120 minutes). Two PLGA copolymers, PLGA 85:15 (77K) (pellet) and PLGA 75:25 (72K) (fine powder), were utilised. The images in Figure 8 show that the pore size of the scaffolds decreased with increasing soaking pressure (a to d for PLGA 85:15, f to i for PLGA 75:25). Singh *et al.* (2004) found the diffusion coefficient and equilibrium concentration of CO_2 in PLGA increased with increasing pressure in an approximately linear relationship. Also, higher pressure was found to increase the dissolution of CO_2 and, as a consequence, the T_g was found to decrease more (Shieh *et al.*, 1996b). Therefore, at a higher pressure, the amount of CO_2 incorporated into the polymers is greater, and hence the substrate is more highly supersaturated upon release of the pressure. These greater super saturation pressures lead to higher nucleation densities and hence smaller pores.

Effect of soaking time. To study the effect of soaking time on scaffold formation, the scaffolds were fabricated using a pellet sample PLGA 85:15 (Figure 8e) and a powder sample PLGA 75:25 (Figure 8j) at a pressure of 100 bar and a soaking time of 4 hours; other processing conditions remained constant as those used during the 1 hour soak, *i.e.* T = 35°C, FT = 20 minutes and VT = 120 minutes. Compared to the scaffolds produced with a shorter soaking time (1 hour) (Figure 8b,g), the scaffolds produced after a longer soaking time clearly show a tight distribution of smaller pores rather than the broader distribution of large pores. As discussed in the previous section, CO_2 might not be distributed uniformly throughout the swollen polymers within a short soaking time (1 hour) at low pressures, which may lead to a CO_2 concentration which is higher at the region close to the interface with CO_2 than inside the bulk of the polymer. However, a longer soaking time allows CO_2 to more efficiently diffuse into the polymers and distribute uniformly, leading to more uniform porous scaffolds after venting (Figure 8e, j). Moreover, the equilibrium time required also depends on the nature of the sample, i.e. sample size and sample form based upon the surface area/volume ratio (Shieh *et al.,* 1996a). Shorter equilibrium times are needed for the powder sample (Figure 8, PLGA 75:25) than the pellet sample (Figure 8, PLGA 85:15).

Conclusions

The pore size and structure of the $P_{DL}LA$ and PLGA porous scaffolds produced using $scCO_2$ can be tailored by altering the processing conditions. A higher pressure and a longer soaking time allowed more CO_2 molecules to diffuse into the polymer matrix, leading to a higher nucleation density and hence the production of smaller pores. Higher temperatures produced foams with larger pores because increased diffusion rates facilitated pore growth. In addition, reducing the rate of depressurisation allowed a longer period for pore growth and therefore larger pores were formed than with rapid depressurisation. The pore size of scaffolds also decreased with increasing glycolic acid content in the PLGA copolymers. This knowledge empowers the definition of processing conditions to tailor the pore size and structure of scaffolds for potential application as controlled release devices for growth factor delivery in Tissue Engineering.

Acknowledgements

We gratefully acknowledge EPSRC for funding on this Grand Challenge project. We also thank Dr P. Ginty, Dr M. Heyde, Mr S. Pygall and Dr C. Melia, Mr R. G. M. Wilson, Mr P. A. Fields and Mr M. P. Dellar for scientific discussions and technical support. S.M.H. is a Royal Society Wolfson Research Merit Award holder.

References

Arora KA, Lesser AJ, McCarthy TJ (1998) Preparation and characterization of microcellular polystyrene foams processed in supercritical carbon dioxide. Macromolecules **31**: 4614-4620.

Barry JJA, Gidda HS, Scotchford CA, Howdle SM (2004) Porous methacrylate scaffolds: supercritical fluid fabrication and *in vitro* chondrocyte responses. Biomaterials **25**: 3559-3568.

Barry JJA, Silva M, Popov VK, Shakesheff KM, Howdle SM (2006) Supercritical carbon dioxide: putting the fizz into biomaterials. Phil Trans Royal Soc A-Math Phys Engg Sci **364**: 249-261.

Cooper AI (2001) Recent developments in materials synthesis and processing using supercritical CO_2. Adv Mater **13**: 1111-1114.

Furno F, Licence P, Howdle SM, Poliakoff M (2003) Recent developments in the use of supercritical CO_2 in synthetic organic chemistry. Act Chim: **4-5**: 62-66.

Goel SK, Beckman EJ (1994a) Generation of microcellular polymeric Foams using supercritical carbon-

dioxide. 1. Effect of pressure and temperature on nucleation. Polymer Engg Sci **34**: 1137-1147.

Goel SK, Beckman EJ (1994b) Generation of microcellular polymeric foams using supercritical carbon-dioxide. II. Cell-growth and skin formation. Polymer Engg and Sci **34**: 1148-1156.

Harris LD, Kim BS, Mooney DJ (1998) Open pore biodegradable matrices formed with gas foaming. J Biomed Mater Res **42**: 396-402.

Heyde M, Partridge K, Howdle S, Oreffo R, Garnett M, Shakesheff K (2007) Development of a slow non-viral DNA release system from P_{DL}LA scaffolds fabricated using a supercritical CO_2 technique. Biotechnol Bioeng **98**: 679-693.

Hile DD, Amirpour ML, Akgerman A, Pishko MV (2000) Active growth factor delivery from poly(D,L-lactide-co- glycolide) foams prepared in supercritical CO_2. J Controlled Rel **66**: 177-185.

Hile DD, Pishko MV (2004) Solvent-free protein encapsulation within biodegradable polymer foams. Drug Delivery **11**: 287-293.

Hou QP, Grijpma DW, Feijen J (2003) Porous polymeric structures for tissue engineering prepared by a coagulation, compression moulding and salt leaching technique. Biomaterials **24**: 1937-1947.

Howard D, Partridge K, Yang XB, Clarke NMP, Okubo Y, Bessho K, Howdle SM, Shakesheff KM, Oreffo ROC (2002) Immunoselection and adenoviral genetic modulation of human osteoprogenitors: *in vivo* bone formation on PLA scaffold. Bioch Biophys Res Commun **299**: 208-215.

Howdle SM, Watson MS, Whitaker MJ, Popov VK, Davies MC, Mandel FS, Wang JD, Shakesheff KM (2001) Supercritical fluid mixing: preparation of thermally sensitive polymer composites containing bioactive materials. Chem Commun **1**: 109-110.

Hutmacher DW (2000) Scaffolds in tissue engineering bone and cartilage. Biomaterials **21**: 2529-2543.

Jang JH, Shea LD (2003) Controllable delivery of non-viral DNA from porous scaffolds. J Controlled Rel **86**: 157-168.

Kazarian SG, Vincent MF, Eckert CA (1996) Infrared cell for supercritical fluid-polymer interactions. Rev Sci Instr **67**: 1586-1589.

Kim TK, Yoon JJ, Lee DS, Park TG (2006) Gas foamed open porous biodegradable polymeric microspheres. Biomaterials **27**: 152-159.

Langer R (1998) Drug delivery and targeting. Nature **392**: 5-10.

Liu DH, Tomasko DL (2007) Carbon dioxide sorption and dilation of poly(lactide-co-glycolide). J Supercrit Fluids **39**: 416-425.

Lu L, Peter SJ, Lyman MD, Lai HL, Leite SM, Tamada JA, Uyama S, Vacanti JP, Langer R, Mikos AG (2000) *In vitro* and *in vivo* degradation of porous poly(DL-lactic-co-glycolic acid) foams. Biomaterials **21**: 1837-1845.

Mooney DJ, Baldwin DF, Suh NP, Vacanti LP, Langer R (1996) Novel approach to fabricate porous sponges of poly(D,L-lactic- co-glycolic acid) without the use of organic solvents. Biomaterials **17**: 1417-1422.

Murphy WL, Peters MC, Kohn DH, Mooney DJ (2000) Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering. Biomaterials **21**: 2521-2527.

Murphy WL, Dennis RG, Kileny JL, Mooney DJ (2002) Salt fusion: An approach to improve pore interconnectivity within tissue engineering scaffolds. Tissue Engg **8**: 43-52.

Nalawade SP, Picchioni F, Marsman JH, Janssen L (2006) The FT-IR studies of the interactions of CO_2 and polymers having different chain groups. J Supercrit Fluids **36**: 236-244.

Nam YS, Yoon JJ, Park TG (2000) A novel fabrication method of macroporous biodegradable polymer scaffolds using gas foaming salt as a porogen additive. J Biomed Mater Res **53**: 1-7.

Partridge K, Yang XB, Clarke NMP, Okubo Y, Bessho K, Sebald W, Howdle SM, Shakesheff KM, Oreffo ROC (2002) Adenoviral BMP-2 gene transfer in mesenchymal stem cells: *In vitro* and *in vivo* bone formation on biodegradable polymer scaffolds. Biochem Biophys Res Commun **292**: 144-152.

Oliveira NS, Dorgan J, Coutinho JAP, Ferreira A, Daridon JL, Marrucho IM (2006a) Gas solubility of carbon dioxide in poly(lactic acid) at high pressures. J Polymer Sci B-Polymer Phys **44**: 1010-1019.

Oliveira NS, Goncalves CM, Coutinho JAP, Ferreira A, Dorgan J, Marrucho IM (2006b) Carbon dioxide, ethylene and water vapor sorption in poly(lactic acid). Fluid Phase Equil **250**: 116-124.

Quirk RA, France RM, Shakesheff KM, Howdle SM (2004) Supercritical fluid technologies and tissue engineering scaffolds. Curr Opin Solid State Mater Sci **8**: 313-321.

Riddle KW, Mooney DJ (2004) Role of poly(lactide-co-glycolide) particle size on gas-foamed scaffolds. J Biomaterials Sci-Polymer Ed **15**: 1561-1570.

Royer JR, DeSimone JM, Khan SA (1999) Carbon dioxide-induced swelling of poly(dimethylsiloxane). Macromolecules **32**: 8965-8973.

Sheridan MH, Shea LD, Peters MC, Mooney DJ (2000) Bioadsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery. J Controlled Rel **64**: 91-102.

Shieh YT, Su JH, Manivannan G, Lee PHC, Sawan SP, Spall WD (1996a) Interaction of supercritical carbon dioxide with polymers. 1. Crystalline polymers. J Appl Polymer Sci **59**: 695-705.

Shieh YT, Su JH, Manivannan G, Lee PHC, Sawan SP, Spall WD (1996b) Interaction of supercritical carbon dioxide with polymers. 2. Amorphous polymers. J Appl Polymer Sci **59**: 707-717.

Singh L, Kumar V, Ratner BD (2004) Generation of porous microcellular 85/15 poly ((DL)-lactide-co-glycolide) foams for biomedical applications. Biomaterials 25: 2611-2617.

Sosnowski S, Wozniak P, Lewandowska-Szumiel M (2006) Polyester scaffolds with bimodal pore size distribution for tissue engineering. Macromol Biosc **6**: 425-434.

Sproule TL, Lee JA, Li HB, Lannutti JJ, Tomasko DL (2004) Bioactive polymer surfaces *via* supercritical fluids. J Supercrit Fluids **28**: 241-248.

Stafford CM, Russell TP, McCarthy TJ (1999) Expansion of polystyrene using supercritical carbon dioxide: Effects of molecular weight, polydispersity, and low molecular weight components. Macromolecules **32**: 7610-7616.

Tomasko DL, Li HB, Liu DH, Han XM, Wingert MJ, Lee LJ, Koelling KW (2003) A review of CO_2 applications in the processing of polymers. Industr Engg Chem Res **42**: 6431-6456.

Watson MS, Whitaker MJ, Howdle SM, Shakesheff KM (2002) Incorporation of proteins into polymer materials by a novel supercritical fluid processing method. Adv Mater **14**: 1802-1804.

Woods HM, Silva M, Nouvel C, Shakesheff KM, Howdle SM (2004) Materials processing in supercritical carbon dioxide: surfactants, polymers and biomaterials. J Mater Chem **14**: 1663-1678.

Yang XB, Green D, Roach HI, Anderson HC, Howdle SM, Shakesheff KM, Oreffo ROC (2006) The effect of an admix of bone morphogenetic proteins on human osteoprogenitor activity *in vitro* and *in vivo*. Tissue Engg **12**: 1002-1003.

Yang XBB, Whitaker MJ, Sebald W, Clarke N, Howdle SM, Shakesheff KM, Oreffo ROC (2004) Human osteoprogenitor bone formation using encapsulated bone morphogenetic protein 2 in porous polymer scaffolds. Tissue Engg **10**: 1037-1045.

Discussion with Reviewers

J. Darr: Why did you select this pressure and temperature range for your experiments?
Authors: The temperature range for our experiments (5-55°C) is selected to be close to ambient temperature in order to limit denaturation of the incorporated bioactives. The determination of the pressure range for our experiments is based on our previous published results. It was found that the use of a high pressure (170-230 bar) can dramatically reduce the prolonged soaking time to 2 hours and produce scaffolds with desired pore size and porosity. However, there is no doubt that using a lower processing pressure is more attractive from both a practical and an economical point of view. Therefore, we selected 230 bar as the highest pressure for our experiments, while the lowest pressure (60 bar) was chosen as a point below the CO_2 critical pressure (73.8 bar).

J. Darr: Does it make any difference to pore sizes/distributions if it is above the critical point?
Authors: The key in a gas foaming process is pore nucleation and growth, which determines the final porous structure (pore size/distribution, porosity, and interconnectivity). This pore nucleation and growth are mainly influenced by the amount of CO_2 dissolved in the polymer, and the rate of CO_2 diffusing within and escaping from the polymer. The $scCO_2$ has a higher density than gaseous CO_2 and a higher diffusivity than liquid CO_2. Therefore, it certainly does affect the formation of a porous structure if it is above or below the critical point. However, the final porous structures of the resultant scaffolds are influenced by many fabrication conditions as well as pressure and temperature, such as soaking time and venting rate and type of polymer.

J. Darr: Did you look at any other molecular weights for the polymers and how would this affect pore sizes etc?
Authors: Yes. We have investigated the effect of molecular weight on morphology of $P_{DL}LA$ scaffolds. The results will be reported in a follow-up paper, which is in the final stage of drafting.

J. Darr: How does porosity affect drug release from these materials?
Authors: The pore structure of scaffolds, including pore size, porosity and interconnectivity, strongly influences cell growth behaviour and drug release profile. A high porosity and interconnectivity within scaffolds could enhance drug release from the matrix. Our research group has reported the studies on BMP-2 growth factor release (Yang *et al*., 2004) and cell growth behaviour using $P_{DL}LA$ scaffolds produced by this $scCO_2$ foaming technique. The research on using PLGA scaffolds for growth factor release is currently on-going at Nottingham.

P. Layrolle: The authors mentioned several times the solubility of $scCO_2$ into the polymers. Is $scCO_2$ fluid not a solvent for polymers? Then, the solubility of polymers into $scCO_2$ should be considered. Please comment.
Authors: PLA and PLGA polymers have negligible solubility in $scCO_2$ under the conditions employed in this research. Indeed, others (Conway *et al*., 2001) have shown that to solubilise even low molecular weight PLA in $scCO_2$ requires extremely forcing conditions.

P. Layrolle: It was found that high pressure and long soaking times increased the diffusion of $scCO_2$ into the polymer matrix leading to numerous nucleation sites and thus, produced numerous small pores. Where the nucleation of CO_2 gas takes place in the polymer matrix? Does the nucleation sites relate to the chemistry or chain length of polymers?
Authors: The number of nucleation sites is determined by the concentration and the solubility of CO_2 in the polymers, as well as the rate of depressurization. Due to depressurization, the polymer/CO_2 mixture reaches saturation, at which the CO_2 starts escaping from the polymer as gas. This phase separation results in the nucleation, which takes place at the interface of polymer matrix and CO_2 gas phase. The nucleation sites might relate to the chemistry or chain length of polymers due to the interaction between CO_2 and the polymer. However, we don't have evidence to support this hypothesis yet.

P. Layrolle: High temperatures produce scaffolds with large pores due to high diffusion of CO_2. Would it be possible to oscillate the system in temperature to produce

scaffolds with bimodal pore size? Bimodal pore size might be of interest for sorting cells and molecules. Please comment.
Authors: This paper has demonstrated that pore size and structure of the $P_{DL}LA$ and PLGA scaffolds can be tailored by altering processing conditions. The knowledge contributed by this research is aimed at allowing us to define the processing conditions and to tailor the pore size and structure of scaffolds for potential applications as controlled release devices for growth factor delivery. It might well be possible to produce scaffolds with bimodal pore size by a two-step venting processing either using two temperatures or perhaps two venting rates.

P. Layrolle: The pore size interconnectivity in scaffolds is extremely important for permeability of body fluids, cells and tissues. However, this parameter has not been investigated in the present study. Please comment.
Authors: The interconnectivity of scaffolds is one of the most important characteristics in terms of biological applications. SEM images can be used to demonstrate the interconnectivity of porous scaffolds qualitatively. In our paper, the SEM images have been used to demonstrate a more open and interconnected pore structure for $P_{DL}LA$ scaffold than for PLGA scaffolds (Figure 2).

P. Layrolle: Liu *et al*. (2007) have shown by FTIR spectroscopy that CO_2 molecules interact with carbonyl group in polyesters. In the present study, the pore size decreased with the GA content in PLAGA polymers. The authors postulated that it may be due to an extra methyl group in GA structure leading to steric hindrance diminishing the interaction between CO_2 and carbonyl groups or that would create more free volume between chains due to a steric effect. How this possible steric hindrance could be shown? Would it possible to use molecular dynamic simulations?
Authors: We don't have expertise on molecular dynamic simulations. However, we agree with the reviewer that a fundamental study at the molecular level using molecular dynamic simulations could be a feasible approach for the illustration of steric hindrance and free volume theory.

P. Layrolle: The authors would like to use these foamed polymers for releasing growth factors. What are the growth factors that they would like to introduce? How are the growth factors loaded and where are they located in the polymers using $scCO_2$ processing? How the growth factor will be released from the polymer matrix? Is it by diffusion, degradation or cellular activity?
Authors: BMP2 growth factor is one of our major interests. It can be loaded easily by mixing with the powdered polymer in the mould, and then the polymer loaded mould is placed in the high pressure autoclave for plasticization and foaming using CO_2 under defined conditions. The growth factor is encapsulated within the polymer matrix and released from the matrix by both diffusion and degradation. Many more details of this process and the *in vivo* data we have obtained can be seen in our joint publications with collaborator Professor R.O.C. Oreffo (University of Southampton).

P. Layrolle: I would be happy to see some release experiments using growth factor or the behaviour of cells into these porous scaffolds.
Authors: Our research group has reported studies on growth factor release and cell growth behaviour using $P_{DL}LA$ scaffolds produced by $scCO_2$ foaming. This includes BMP-2 growth factor delivery (Yang *et al*., 2004), where bone formation was observed due to the release of the osteoinductive protein BMP-2 from $P_{DL}LA$ scaffolds both *in vitro* and *in vivo* (Yang *et al*., 2004; Yang *et al*., 2006). These scaffolds have also been used to study adenoviral gene transfer into primary human bone marrow osteoprogenitor cells (Partridge *et al*., 2002; Howard *et al*., 2002). Very recently, polyamidoamine (PAA)/DNA complexes have been incorporated into supercritical $P_{DL}LA$ scaffolds; these exhibited a slow release and extended gene expression profile (Heyde *et al*., 2007). The study of growth factor release using PLGA scaffolds is currently on-going in our research group.

T. Buckland: Please justify use of μCT and the lack of standard deviations for total porosity measurements given the importance of this parameter to biological performance.
Authors: Our experimental results indicate a reproducible 2-3 % of standard deviation on porosity evaluations by using μCT technique. μCT is a powerful technique for the evaluation of porosity by using 3-D construction; by contrast, SEM technique only allows 2-D image analysis. The standard deviation of porosity obtained by μCT has been added in the revised manuscript (Table 2).

Additional Reference

Conway SE, Byun HS, McHugh MA Wang JD, Mandel FS (2001) Poly(lactide-co-glycolide) solution behavior in supercritical CO_2, CHF_3, and $CHClF_2$. J Appl Polymer Sci **80**: 1155-1161.