Systematic selection of solvents for the fabrication of 3D combined macro- and microporous polymeric scaffolds for soft tissue engineering

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Abstract—In this study, we investigate the fabrication of 3D porous poly(lactic-co-glycolic acid) (PLGA) scaffolds using the thermally-induced phase separation technique. The current study focuses on the selection of alternative solvents for this process using a number of criteria, including predicted solubility, toxicity, removability and processability. Solvents were removed via either vacuum freeze-drying or leaching, depending on their physical properties. The residual solvent was tested using gas chromatography-mass spectrometry. A large range of porous, highly interconnected scaffold architectures with tunable pore size and alignment was obtained, including combined macro- and microporous structures and an entirely novel ‘porous-fibre’ structure. The morphological features of the most promising poly(lactic-co-glycolic acid) scaffolds were analysed via scanning electron microscopy and X-ray micro-computed tomography in both two and three dimensions. The Young’s moduli of the scaffolds under conditions of temperature, pH and ionic strength similar to those found in the body were tested and were found to be highly dependent on the architectures.

Key words: Microstructure; scaffold; tissue engineering.

INTRODUCTION

Current research in the field of tissue engineering is focused on the development of appropriate strategies for repair and regeneration of biological tissues. Artificial three-dimensional (3D) polymeric scaffolds serve as a physical support to provide tissues with the appropriate architecture for in vitro cell culture, as well as in vivo

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tissue regeneration. The design and manufacture of 3D polymeric scaffolds which mimic the natural extracellular matrix (ECM) found within the body are crucial to the success of tissue engineering.

A significant challenge in the production of 3D polymeric scaffolds for tissue engineering is the development of scaffolds with arbitrary shape and size, yet with tailored micro- and macroporous architecture and mechanical properties [1]. This is believed to be a crucial step in promoting controlled vascularisation and tissue growth within the host environment [2].

Pore size and pore structure in polymeric scaffolds may dramatically influence cell behavior, and this effect may be exploited in tissue engineering [3]. For tissue engineering scaffolds, a macroporous open cellular structure is often required with pore sizes of 100–300 µm for cell penetration [4]. Microcellular foams with pore diameters ranging from 10 to 100 µm have been shown to give optimal in-growth of fibroblasts for the regeneration of skin [5]. Fibrovascular tissue will invade a device if the pores are larger than approx. 10 µm, and the rate of invasion will increase with the pore size and total porosity of a device [6]. The effect of the pore architecture on growth of various cell types has been reviewed in Yang et al. [7].

Numerous processing techniques for producing 3D scaffolds from various biodegradable polymers have been described in the literature. These are reviewed in, for example, Yang et al. [7], Hutmacher [8] and Mikos et al. [9]. Among a plethora of techniques, thermally-induced phase separation (TIPS) uses thermal energy as a driving force to induce phase separation [10]. Solid–liquid or liquid–liquid phase separation of a polymer solution is induced by lowering the solution temperature. Subsequent removal of the solvent is achieved by sublimation or, more recently, leaching into a non-solvent for the polymer, leaving a highly porous structure. This technique is employed commercially to produce microporous membranes for filtration and plasmapheresis [11]. In the past decade, many researchers have been using TIPS to make biodegradable tissue scaffolds [12–14].

The advantages of the TIPS technique lie in its capability to rapidly produce highly porous, interconnected structures [15] of virtually any shape and size. Slight changes in the processing parameters, such as the type of polymer, polymer concentration, solvent/non-solvent ratio, or the thermal quenching strategy [16] significantly affect the resultant architecture of the porous scaffold. Thus, a range of porous structures can be easily obtained. However, most of the solvents typically used at present for the production of poly(lactic-co-glycolic acid) (PLGA) tissue-engineering scaffolds via TIPS are toxic and/or carcinogenic and, hence, are of questionable utility in a clinical application.

While a large number of small compounds exist having structures indicating that they are good solvents for PLGA [17], the number of solvents with physical properties suitable for the TIPS process is much smaller. In order to facilitate convenient processing, the melting point (MP) of the solvent should be within a range that can be easily reached by standard laboratory equipment, that is, no lower than −20°C and preferably above 0°C. The solvents used within this study were selected from
### Table 1.
Physical properties of selected (italics) and commonly used solvents

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Structure</th>
<th>MP (°C)</th>
<th>BP (°C)</th>
<th>PV (mbar)</th>
<th>Miscibility&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Toxic effects&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td><img src="image" alt="structure" /></td>
<td>77</td>
<td>218</td>
<td>0.04</td>
<td>−</td>
<td>TMRI</td>
<td>0.49</td>
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<tr>
<td>Dimethyl oxalate (DMO)</td>
<td><img src="image" alt="structure" /></td>
<td>50–53</td>
<td>163</td>
<td>&lt;0.13</td>
<td>/</td>
<td>C</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Ethylene carbonate (EC)</td>
<td><img src="image" alt="structure" /></td>
<td>37–39</td>
<td>243–244</td>
<td>&lt;0.13</td>
<td>+</td>
<td>I</td>
<td>10</td>
</tr>
<tr>
<td>N-Methylacetamide (NMA)</td>
<td><img src="image" alt="structure" /></td>
<td>26–28</td>
<td>204–206</td>
<td>0.59</td>
<td>+</td>
<td>MR</td>
<td>5</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td><img src="image" alt="structure" /></td>
<td>18.4</td>
<td>189</td>
<td>0.56</td>
<td>+</td>
<td>TDMRI</td>
<td>14.5</td>
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<tr>
<td>Acetic acid (AA)</td>
<td><img src="image" alt="structure" /></td>
<td>16.2</td>
<td>117–118</td>
<td>15.2</td>
<td>+</td>
<td>MRIC</td>
<td>3.3</td>
</tr>
<tr>
<td>1,4-Dioxane (DO)</td>
<td><img src="image" alt="structure" /></td>
<td>11.5–12</td>
<td>100–102</td>
<td>53.3</td>
<td>+</td>
<td>TMRI</td>
<td>4.2</td>
</tr>
<tr>
<td>Benzene</td>
<td><img src="image" alt="structure" /></td>
<td>5.5</td>
<td>80</td>
<td>134</td>
<td>−</td>
<td>TDMRI</td>
<td>0.93</td>
</tr>
<tr>
<td>Dimethyl carbonate (DMC)</td>
<td><img src="image" alt="structure" /></td>
<td>2–4</td>
<td>90</td>
<td>26.7</td>
<td>−</td>
<td>−</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup>T, tumoren; D, drug; M, mutagen; R, reproductive effector; I, irritant; C, corrosive. Boldface, strong effect.

<sup>b</sup>Lethal dose 50% mortality oral in rat (g/kg).

<sup>c</sup>Miscibility with water. +, miscible; −, immiscible; /, partially miscible.

the larger set of potential solvents primarily on this basis. In order to be suitable for removal via the vacuum freeze-drying method, the vapor pressure of the solvent needs to be as high as possible. As can be seen from Table 1, the vapor pressures (at 25°C) of acetic acid (AA), dioxane (DO) and dimethylcarbonate (DMC) are similar, and experiments have shown that at least 99% removal of these solvents may be achieved by vacuum freeze-drying at 0°C and 10⁻² mbar within 24 h.

The current study focuses on the selection of alternative solvents for the creation of PLGA scaffolds via the TIPS process according to a number of criteria, including predicted solubility, toxicity, removability and processability. Solvents were removed via either vacuum freeze-drying or a leaching process depending on their physical properties. The amount of residual solvent was tested using gas chromatography-mass spectrometry (GC-MS). The morphological features, including pore size, porosity, surface area, surface/volume ratio and degree of
anisotropy of the most promising scaffolds produced were analyzed via scanning electron microscopy (SEM) and X-ray micro-computed tomography (micro-CT) in both two and three dimensions. The Young’s moduli of the scaffolds under conditions of temperature, pH and ionic strength similar to those found in the body were tested.

MATERIALS AND METHODS

Materials

Biomedical grade, white granular solid 75/25 poly(D,L-lactic-co-glycolic acid) (PLGA) (approx. molecular mass 100 kDa) with an inherent viscosity of 0.69 dl/g in chloroform at 30°C was obtained from Birmingham Polymers (Birmingham, AL, USA) [18]. All solvents used in this study, dimethyl oxalate (DMO), ethylene carbonate (EC), N-methylacetamide (NMA), dimethyl sulfoxide (DMSO), acetic acid (AA), 1,4-dioxane (DO), dimethyl carbonate (DMC), deuterated chloroform (d-CF), dichloromethane (DCM), hexane and ethanol, were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was used in the leaching and washing processes.

Preparation of porous scaffolds

The 3D PLGA scaffolds were fabricated using a TIPS technique similar to that described previously [19]. Physical properties of selected and commonly used solvents are summarised in Table 1. Briefly, PLGA pellets were dissolved in the selected solvents to a final concentration of 5% (w/v) at a suitable temperature. Of each of these solutions, 8 ml was placed in 17-mm internal diameter glass vials, and the vials were capped. These were immersed vertically in a water bath (FRIGOMIX® U, Braun Biotech International, Germany) containing a 50% ethylene glycol/water mixture, attached to a computer with control software developed in-house. Solid–liquid phase separation was induced by decreasing the temperature of the PLGA solution from 15°C above the solvent melting point (MP) to 20°C below the MP using two different cooling regimes. In the first case (slow quench, SQ), the bath temperature was decreased from the high to the low temperature at a constant rate of 0.1°C/min. In the second case (quick quench, QQ), the water bath temperature was held constant at the low temperature to induce a steep temperature gradient. The solidified solvent was subsequently removed via vacuum freeze-drying or leaching depending on the solvent vapor pressure and MP, leaving a 3D highly porous polymer structure.

Centerline temperature measurement

The temperature at the centre of the PLGA solution (centerline temperature) during the cooling process was measured by inserting a 1.6-mm diameter type-T
Selection of solvents for fabrication of scaffolds

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copper/copper-nickel thermocouple probe (RS, UK) vertically into the centre of the casting mould to half of the depth of the solution. The top of the solution was covered by four glass microscope slides closely surrounding the probe. The reading from the thermocouple was recorded using a multimeter (Hewlett–Packard, Model 34401A) connected to a PC via a RS-232 serial connection, and further processed using software developed in-house.

Solvent removal

Vacuum freeze-drying. For solidified PLGA solutions made using AA, DO and DMC, due to their relatively high vapor pressure (Table 1), the solvents were removed by vacuum-drying at $10^{-3}$ mbar for 72 h in a vessel surrounded by an ice/water slurry.

Leaching. Solidified scaffolds made using DMO, EC, NMA, DMSO, AA and DO were further cooled by dipping in liquid nitrogen to prevent melting and removed from their moulds by carefully breaking the glass. These were then placed in a coarsely woven nylon mesh bag and suspended in a 2-l beaker containing a water/ice mixture, so that the extracted solvent drained under gravity due to the higher density of the solvent/water mixture compared to pure water. The ice/water mixture was changed every 2 h until the solvent stopped visibly draining. The time required for this step varied from 6 h for the most water-miscible solvent (EC) to 24 h for the least miscible (DMO). At this point the water was changed once more, and a magnetic stirrer bar added to the beaker. The scaffolds were soaked for a further 24 h at room temperature with stirring. Scaffolds made using DMC were leached via an analogous method in hexane held at a temperature of $-18^\circ$C. Residual hexane was removed by vacuuming at $10^{-3}$ mbar and room temperature for 24 h.

Washing of freeze-dried scaffolds

Scaffolds prepared via vacuum freeze-drying were further washed to reduce the residual solvent to levels as low as possible. The scaffolds were placed in a custom made glass basket and dipped in ethanol for 10 min. The basket was then transferred to a 500-ml beaker containing distilled water with a magnetic stirrer and left at room temperature for 6 h, changing the water at 2-h intervals. The washed PLGA scaffolds were placed on a Petri dish lined with lint-free tissue (Kimwipes™) and allowed to stand overnight to air-dry. Residual water was removed by vacuuming at $10^{-3}$ mbar and room temperature for 12 h.

SEM analysis

The morphology of 3D PLGA scaffolds was observed using a SEM (XL30, Philips, The Netherlands). A 2-mm-thick radial section was removed from each sample.
at the base and at half-height using a surgical blade (Fig. 1). The half-height cross-sectional surfaces and the bottom (outside) surfaces were coated with gold in a sputter coater (Dynavac mini coater, USA) under an argon atmosphere using a sputter current of 60 mA. The coated samples were observed under SEM at an accelerating voltage of 20 kV.

**Micro-CT analysis**

The 3D macroporous internal structure of the obtained scaffolds was characterised non-destructively using a desk-top X-ray micro-CT system, Skyscan1072_100 kV (Skyscan, Belgium), with an air-cooled tungsten X-ray tube set at 50 kV and 181 µA. The system consists of the combination of an X-ray shadow microscopic system and a computer with tomographic reconstruction and analysis software. It allows one to obtain transmission images and reconstruct cross-sections or a 3D reconstruction of the internal microstructure. Based on the reconstructed 3D structures, estimates of porosity, surface-area-to-volume ratio and degree of anisotropy (DA) were calculated using ANT software (Skyscan, Belgium) [20]. Data are presented as mean ± standard deviation. A DA value of zero indicates a completely isotropic structure, while a DA of one indicates a highly aligned, anisotropic structure.

A sample approximately 2–3 mm × 2–3 mm × 40 mm was removed from each scaffold as shown in Fig. 1 to fit the sample holder. The samples were then scanned with the micro-CT at 5 µm spatial resolution with an integration time of 1.7 ms. The samples were set to rotate from 0 to 180° at increments of 0.45°.

**Mechanical testing**

The initial stress (σ) and strain (τ) of the obtained cylindrical 3D PLGA scaffolds were determined using a texture analyser (TA-XT2™, Stable Micro Systems, UK).
The initial or Young’s modulus \( (E) \) was obtained by Hooke’s law:

\[
E = \frac{\sigma}{\tau}.
\]

In order to understand the effect of changes in the internal scaffold architecture on the mechanical properties, the outside layer (a ‘skin’) was removed. The original cylindrical scaffolds were dipped into liquid nitrogen and a cubic sample was obtained from the position shown in Fig. 1 using a surgical blade to remove the surrounding polymer without damage to the internal structure. Samples were then wet with phosphate-buffered saline (PBS) solution at pH 7.4 via a pressure-swing method. Briefly, the scaffolds were anchored beneath the surface of the PBS and degassed under vacuum for 10 min. The vacuum was then slowly released, driving the PBS into the scaffolds. The scaffolds were then incubated in PBS at 37°C overnight to simulate in vivo conditions before testing. Three to four samples for each group were tested. Data are presented as mean ± standard deviation.

**Residual solvent estimation via GC-MS**

Approximately 10-mg samples from the central region of scaffolds were dissolved in 10 ml of either DCM (DMC scaffolds) or d-CF (all other scaffolds). The DMC samples were dissolved in DCM due to the very small difference in retention time between d-CF and DMC. A 5-µl aliquot of the dissolved sample was injected into a GC column (ZB-5 5% Phenyl polysiloxane, Zebron, Phenomenex, Australia) installed in a GC-MS instrument (GC/MS-QP5050, Shimadzu, Japan) with an acceleration voltage in the mass spectrometer of 2.0 kV.

**RESULTS**

**Solution cooling profiles**

For all the selected solvents, the temperature at the centre of the PLGA solution was measured under both SQ and QQ conditions. The temperature profiles collected are shown in Figs 2 and 3, respectively. Under SQ conditions (Fig. 2) the temperature at the centerline was within 1°C of the temperature of the cooling bath in the absence of phase changes, indicating a uniform temperature profile across the solution. The crystallization plateaus of the PLGA solutions were very near to the melting temperatures of the corresponding pure solvents (Table 1) and were independent of the quenching conditions, indicating that the existence of a small amount of PLGA (5 %, w/v) in the solvent has almost no effect on the solvent melting temperature.

Under SQ conditions, a significant amount of supercooling was observed at the centreline for all solvents (Fig. 2); however, the degree of supercooling varied widely between solvents. DMO, EC and AA in particular showed very significant supercooling of 10°C to 12°C below their melting temperatures. NMA, DMSO and
DO showed relatively less supercooling (approx. 5°C), while DMC supercooled by <2°C.

Under QQ conditions (Fig. 3), only EC and AA showed supercooling at the centreline of the solution. EC in particular showed a very large degree of supercooling of approx. 17°C below the solvent melting temperature.

In both cases DO showed two temperature plateaus at 11.5°C and 0°C, corresponding to the crystallisation and crystal (I)–crystal (II) phase transitions, respectively [21].

Architecture — gross examination

For all solvents, except DMC and NMA under SQ conditions, a cone-shaped depression appeared in the top of the scaffolds due to shrinkage of the solvent during the freezing process. The depth of this cone was generally higher for scaffolds prepared under QQ conditions than for those prepared under SQ conditions.
Interestingly, scaffolds made using 10% (w/v) PLGA in NMA under SQ conditions showed a slight bump in the centre, indicating an expansion of the solvent during freezing; however, under QQ conditions there was a cone with a depth of approx. 30% of the scaffold height. Scaffolds made using DMC showed no cone, indicating little or no shrinkage on freezing.

Scaffolds leached in water generally shrank slightly during the leaching process by about 5–10% in their diameter, with slight wrinkling at the outer surface. Scaffolds made using AA and 5% (w/v) PLGA in NMA under SQ conditions lost all mechanical integrity during the leaching process. Scaffolds made from AA under QQ conditions retained some structure during leaching, but were extremely fragile. The scaffolds made from 5% (w/v) PLGA in NMA under QQ conditions spread out somewhat during leaching, with a final structure resembling cotton wool. Scaffolds made using DMO were very difficult to leach due to the low solubility of DMO in water, and were hard, brittle and very fragile after leaching.

Attempts were made to leach the solvents in methanol, ethanol and iso-propyl alcohol at 0°C or −78°C; however, in all cases the polymer structure collapsed and drained as a milky suspension within the leached solvent.

Scaffolds made using DMC showed a shiny, reflective surface, in contrast to scaffolds made using all other solvents, which were a matte white. In particular, SQ DMC scaffolds were translucent, allowing the shape of a dark object placed behind an axially sectioned scaffold to be seen through the scaffold radius.

Architecture — SEM

Figure 4 shows the surface morphology of leached EC, DMSO, DO and DMC scaffolds. The observation of this region of scaffolds made using DMO and AA is shown in the Appendix. In general, scaffolds made under QQ conditions (a, c, e, g) had smaller pores than those made under SQ conditions (b, d, f, h). Depending on the solvent and quenching conditions, maximum pore sizes at the surface ranged from about 50 µm to less than 10 µm for all solvents, except DMC. Under SQ conditions DMC formed an overlapping plate-like structure, with spacing of up to 200 µm. While for most solvents the surface structure appeared quite random, DO showed a relatively highly ordered cellular structure. In all cases the pores were very highly interconnected and open, i.e., the dimensions of the connections between major pores were on the order of the dimensions of the pores themselves.

Figure 5 shows the outer region of the cross-section at half height of leached EC and DMC scaffolds. In general, scaffolds made under QQ conditions (a, c, e) showed directionally distributed, fibre-like structures, with alignment facing to the centre, while scaffolds made under SQ conditions (b, d) showed relatively isotropic structures with pore sizes ranging from approximately 10 µm to 50 µm. The scaffolds made using EC showed a similar interconnected and isotropic structure with similar pore sizes regardless of the quenching conditions. Note that the obvious inhomogeneities in the EC scaffolds appear to be an artifact of the early leaching technique (specifically, they were formed during the change of water in the
leaching bath). Scaffolds leached in later experiments using a continuous leaching system showed essentially isotropic structures throughout (not shown). Scaffolds made using DMC under SQ conditions displayed a structure made up primarily of very large, smooth parallel plates, spaced from 50–500 µm apart, aligned with the direction of heat transfer. Under QQ conditions, the structure was similar; however, the plates were smaller with lower spacing and contained many more defects. All

**Figure 4.** SEM observation of surface morphology of leached EC, DMSO, DO and DMC scaffolds. (a) EC QQ, (b) EC SQ, (c) DMSO QQ, (d) DMSO SQ, (e) DO QQ, (f) DO SQ, (g) DMC QQ, (h) DMC SQ. Scale bar = 200 µm (h), 50 µm (all other images).
Figure 5. SEM observation of the outer region of the cross-section at half height of leached EC and DMC scaffolds. (a) EC QQ, (b) EC SQ, (c) DMC QQ, (d) DMC SQ. Scale bar = 200 µm (d), 500 µm (a, b, c).

scaffolds showed a sharply-defined region, or skin, near the outer edge characterized by pores much smaller than in the bulk.

The observation of the outer region of scaffolds made using other solvents such as DMO, DMSO, DO, AA, as well as the central region of the cross-section at half height of leached scaffolds can be found in the Appendix.
Figure 6. SEM observation of the microporous wall structures formed during the leaching process. (a) DMSO QQ, (b) DMSO SQ, (c) DMC QQ, (d) DMC SQ. Scale bar = 20 µm (a, b), 50 µm (c, d).

Figure 6 shows the observed typical microporous wall structures of scaffolds made using DMSO and DMC. For all other scaffolds leached in water, the walls of the macropores were themselves highly porous, with micropores ranging from <500 nm to a few micrometers forming a honeycomb structure (Appendix, Fig. A4). Correspondingly, the surfaces of the walls displayed high levels of micrometer-scale roughness. For the DMC scaffolds leached into hexane (c, d), the cross-section of the walls showed only a few isolated pinhole-like pores with sizes of a few hundred nanometers. Moreover, the surfaces of these plate-like walls were extremely smooth, with no observable roughness other than the pinholes even at a magnification of >5000× (not shown).

The architecture of the outer region of the cross-section at half height of the vacuumed scaffolds is shown in Fig. 7. As for the leached scaffolds, scaffolds made from DO and DMC (a, c) under QQ conditions had highly anisotropic, directional pores, while scaffolds made from DO under SQ conditions showed random pore structures. Scaffolds made using DMC (c, d) displayed the same layered, sheet-like structure as observed in the hexane-leached samples. While the pores were extensively interconnected in the plane of the sheets, there was
relatively little interconnection in the perpendicular direction. The scaffolds made using AA again showed a highly random structure with highly interconnected pores (not shown); however, the PLGA was only poorly connected by narrow bridges between individual plates.

Further SEM observations of the surface and central region of the cross-section at half height of vacuumed scaffolds can be found in the Appendix.

In contrast to scaffolds made using all other solvents, scaffolds made using NMA displayed a distinctive, novel ‘porous-fiber’ structure, as shown in Fig. 8. Scaffolds made from a 5% solution formed a very loose bundle of these fibers, with fiber diameters of approx. 300 µm and pore sizes up to 50–100 µm within the fibers. Scaffolds made using 10% (w/v) PLGA concentration (c, d) showed a similar structure; however, the fibers were narrower and much more densely packed. In addition, a large number of loose PLGA beads with sizes of a few to 10 µm were observed to be attached to the fiber surfaces in the scaffolds made from 10% PLGA (see Appendix).
Figure 8. SEM observation of the structure formed using NMA as a solvent. (a) 5% (w/v) PLGA, (b) 10% (w/v) PLGA. Scale bar = 500 µm.

Quick Quench

Slow Quench

Figure 9. Micro-CT 3D reconstruction of vacuum freeze-dried PLGA scaffolds. (a) DMC QQ, (b) DMC SQ. The side length of the cubes is approx. 750 µm.

Architecture — micro-CT

Micro-CT 3D reconstruction of PLGA scaffolds was used to display an overview of the macroporous architecture of the scaffolds. The structures determined via micro-CT agreed well with SEM images of scaffolds produced via the same method.

Figure 9 shows examples of reconstructed cubic sections with side lengths of approx. 750 µm from scaffolds made using DMC under quick (Fig. 9a) and slow (Fig. 9b) conditions. The directional architecture of the scaffold made under QQ conditions (Fig. 9a) contrasts strongly with the sheet-like architecture resulting from SQ conditions (Fig. 9b). More examples of reconstructed images from scaffolds made using other solvents can be found in Figs A7 and A8 in the Appendix.

Similar images were obtained for the most promising solvents and quenching strategies (not shown). Estimates of porosity, surface-area/volume ratio and degree
Table 2.
Summary of the morphological features of PLGA scaffolds made during this study

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Quenching conditions</th>
<th>Solvent removal strategy</th>
<th>Morphology&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Porosity (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Surface area/volume (mm&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Degree of anisotropy&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>DMO</td>
<td>QQ</td>
<td>Leach</td>
<td>DI FP</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EC</td>
<td>QQ</td>
<td>Leach</td>
<td>RIP</td>
<td>92 ± 3</td>
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<td>0.31 ± 0.03</td>
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<tr>
<td></td>
<td>SQ</td>
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<td>RIP</td>
<td>92 ± 2</td>
<td>19 ± 2</td>
<td>0.28 ± 0.05</td>
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<td>DTN</td>
<td>93 ± 5</td>
<td>13 ± 4</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>真空</td>
<td>DSN</td>
<td>91 ± 3</td>
<td>22 ± 3</td>
<td>0.55 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>SQ</td>
<td>Leach</td>
<td>DISN</td>
<td>91 ± 5</td>
<td>15 ± 2</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>真空</td>
<td>DISN</td>
<td>95 ± 4</td>
<td>9 ± 1</td>
<td>0.27 ± 0.12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Morphological descriptors from analysis of SEM and micro-CT images. D, directional; I, interconnected; R, random; F, fiber-like; L, ladder-like; T, tube-like; C, cell-like; S, sheet-like; P, microporous walls; N, non-porous walls.

<sup>b</sup>Calculated from micro-CT data.

of anisotropy of the micro-CT samples were calculated using the analysis software provided with the instrument and are listed in Table 2. Interconnectivity was also qualitatively assessed from visual observation of the reconstructions. The calculated porosity of the characterized 5% PLGA scaffolds ranged from approx. 88 to 95%, while the calculated surface-area-to-volume ratio ranged from approx. 8 to 22 mm<sup>-1</sup>. As expected, scaffolds which displayed directional pore structures under SEM analysis had a significantly higher calculated degree of anisotropy than scaffolds with a random or sheet-like structure.

**Mechanical properties**

The Young’s modulus of the scaffolds in the direction of the axis of the original cylindrical mould ranged from 0.02 to 0.7 MPa as shown in Fig. 10 (n = 3–4). For scaffolds made using DO and DMSO, the Young’s modulus was significantly higher for scaffolds made under SQ conditions than for scaffolds made under QQ conditions. For DMC, the inverse was true, while there was no significant difference for scaffolds made using EC. Scaffolds made from DO using vacuum freeze-drying showed an order-of-magnitude increase in modulus compared to leached samples.
Figure 10. Young’s modulus measurement of leached and vacuumed scaffolds under wet conditions.

Table 3. Residual solvent from different processing techniques measured using GC-MS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Residual concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC leached</td>
<td>&lt;5</td>
</tr>
<tr>
<td>DMSO leached</td>
<td>&lt;1</td>
</tr>
<tr>
<td>DO leached</td>
<td>20</td>
</tr>
<tr>
<td>DO vacuumed (unwashed)</td>
<td>29 200</td>
</tr>
<tr>
<td>DO vacuumed (washed)</td>
<td>600</td>
</tr>
<tr>
<td>DMC vacuumed (unwashed)</td>
<td>21</td>
</tr>
<tr>
<td>DMC vacuumed (washed)</td>
<td>14</td>
</tr>
</tbody>
</table>

Residual solvent

The amount of residual solvent within the scaffolds made using a few promising solvents (EC, DMSO, DO and DMC) and different processing methods (leaching and vacuuming) was determined using GC-MS (Table 3). In general, the amount of residual solvent in the leached scaffolds was significantly lower than the vacuumed scaffolds, e.g., the solvent residue in the DO leached scaffold is 30-times lower than in the DO vacuumed and washed sample. For the leached samples, the DMSO sample showed the lowest solvent residue at less than 1 ppm. Among the vacuumed samples, the residual solvent in the washed scaffolds was markedly lower than the unwashed counterparts, depending on the solvents used. The residual solvent in the washed DO scaffolds was more than 40-times lower than in the unwashed scaffolds, while for the scaffolds made from DMC, the residual solvent in the washed scaffolds was two-thirds of that in the unwashed counterparts.
DISCUSSION

Utilizing the principle of ‘like dissolves like’ it may be inferred that PLGA is dissolvable, in general, in solvents of intermediate polarity, e.g., esters, amides, ketones, sulfoxides, sulfones and halocarbons. On the other hand, PLGA is insoluble in non-polar solvents, such as hexane, or strongly polar solvents, such as alcohols or water. Of the aforementioned good solvents, the commonly-used solvents for PLGA form only a very small subset.

Toxicity of any residual solvent is a crucial issue when dealing with substances destined for implantation into human bodies. Under this criterion, the solvents EC and DMC stand out, with no evidence of tumorigenic, mutagenic or reproductive effects found in the literature, and very low acute toxicity as measured by the LD₅₀ (lethal dose 50% mortality oral in rats) [22]. DMSO, NMA and naphthalene also show low acute toxicity; however, there has been some indication of tumorigenic, mutagenic and/or reproductive effects found in the past, albeit at very high dosages [22]. By comparison, benzene and DO, both of which are often reported in the literature in association with TIPS [10, 13, 23], are known to show strong evidence of tumorigenic effects [22].

The directional pore structures generated using DMO, DMSO, DO and DMC under QQ conditions appear characteristic of dendrite formation at the solidification front [22–26]. As the solidification front proceeds, polymer is ejected by the solidifying solvent into the interface between the solid and liquid. If the solidification rate is high enough, the front proceeds faster than the polymer can diffuse away, creating a concentration gradient. Due to the higher polymer concentration at the solid front, the local melting point of the solvent is reduced slightly, a phenomenon known as constitutional supercooling [24]. Random fluctuations in the liquid cause points of slightly lower polymer concentration, leading to a faster growth rate at these points. This faster-growing solid moves further into the bulk solution, hence experiencing an even lower polymer concentration, and still faster growth rate. Over time, a stable ‘mushy region’ is formed, with dendrites of solid solvent surrounded by channels of higher-melting-point solution. Depending on the rate of dendrite growth and the solvent properties, these dendrites may form secondary and/or tertiary branches. As the crystals grow wider, the polymer is forced into the narrow gap between crystals, and eventually precipitates to form a negative image of the dendrite structure.

Under SQ conditions, all solvents displayed some degree of supercooling throughout the solution (Fig. 2). Under these conditions, crystals grow according to the free growth mechanism [24]. Since the driving force for crystallisation is essentially equal in all directions, once a nucleation site appears the crystals sprout in random directions, forming a relatively isotropic network. As cooling continues, the crystals grow further to fill the remaining space, and once again the polymer is forced into a negative image of the crystal structure.

While supercooling is also expected to occur under QQ conditions, for all solvents, except EC and AA, the solution appears to begin to solidify before the temperature towards the middle of the solution drops below the solvent melting
point. The SEM images of the outer section of the scaffolds (Figs 5 and 7) made using these solvents show a thin band with relatively small and random pores. We attribute this band to the supercooling effect. EC and AA supercooled throughout the structure even under QQ conditions; for these solvents, scaffolds made under QQ conditions were essentially identical to those made under SQ conditions.

Interestingly, scaffolds prepared using DMC gave a structure made up of layered, very smooth and relatively thick plates of PLGA, connected periodically by struts. It appears that due to the planar structure of the DMC molecule [25], the DMC preferentially forms planar, rather than dendritic, structures.

Scaffolds made using NMA displayed an entirely different structure to scaffolds made using the other solvents. The structure still appears somewhat dendritic; however, in this case the PLGA appears to form a positive, rather than negative, image of the crystal structure. It is our belief that this is due to the co-crystallisation of PLGA with the NMA. NMA is a highly structured solvent, known to line-up via hydrogen bonding into rigid chains up to 10 molecules in length [26]. Furthermore, NMA is a strong structural analogue of the lactic acid residues in PLGA. Using the molecular modelling package HyperChem® 7 (HyperCube), we have shown that the amide hydrogen in NMA is capable of forming hydrogen bonds with the carbonyl oxygen in PLGA, while the two methyl groups align strongly with the non-polar regions of the PLGA chain (Fig. 11).

This mechanism of structure formation means that there is no strong driving force to concentrate the PLGA into interconnected structures. Rather, the polymer is homogeneously spread throughout the crystals, and clumps together as the NMA is extracted during the leaching process. Some of this polymer forms a continuous network within the individual crystals; however, a significant fraction forms un-connected beads. Indeed, the structures formed using 10% (w/v) PLGA in NMA (Fig. 8) showed a large number of microbeads (see Appendix) with diameters ranging from a few to 10 µm; the structures formed from 5% PLGA

![Figure 11. Ab initio molecular alignment of NMA with lactic acid residue in PLGA.](image-url)
were presumably open enough to allow most of these beads to be washed out during the leaching process. The solvent draining from these scaffolds during leaching had a milky appearance, indicating the presence of suspended particles. These structures, while interesting, do not appear ideal as scaffolds for tissue engineering applications. The microparticles present in large numbers within the structures are smaller than red blood cells and, hence, may be carried into the bloodstream, which is highly undesirable in a tissue engineering situation. In addition, due to the low interconnectivity of the polymer network, the scaffolds are extremely soft and somewhat fragile.

For the leaching process, the leaching agents need to meet a number of criteria. Firstly, they must be a non-solvent for PLGA. Highly polar solvents, such as water and alcohols, and non-polar solvents meet this first criterion. Secondly, the leaching agent must be highly miscible with the solvent used. Thirdly, the PLGA must not be soluble or significantly softened in the mixture of the leaching agent and the solvent. This criterion may be met by decreasing the temperature of the leaching bath to reduce the maximum concentration of solvent in the leachate, and using the gravity-draining technique to quickly remove the solvent-rich leachate. Attempts to use alcohols such as methanol, ethanol and iso-propyl alcohol failed on this criterion. Even when leaching at the temperature of solid CO\textsubscript{2} (−78°C) the PLGA scaffolds collapsed completely during leaching. For all solvents other than DMC, water appears to be the best leaching agent, based on these criteria. For DMC, due to its low miscibility with water and low melting point, the scaffolds were instead leached using hexane at −18°C.

A notable characteristic of the scaffolds made via leaching into water was that the walls of the macropores were themselves highly microporous (Fig. 6). We believe that these pores are due to the transient swelling of the polymer during the leaching process (Fig. 12). As water penetrates and solubilizes the frozen solvent, the mobility of the polymer chains is increased, causing localized swelling. However, as the concentration of water increases, the solubility of the polymer is decreased and the polymer contracts away from the water present within its structure, leaving microscopic voids. This appears to be a highly desirable characteristic, as these micropores may be expected to help in the transport of acidic degradation products out of the polymer, preventing the ‘acid burst’ behaviour seen in denser lactide- and glycolide-based implants due to the autocatalytic effect [27–29]. The surface area/volume ratio of these scaffolds is also obviously greatly increased, possibly offering more opportunity for the attachment of growth factors and other slow-release agents.

The structures obtained during this study are summarized in Table 2. It can be seen that in most cases, the solvent type had very little effect on the final scaffold structure compared to the quenching strategy. Only solvents that displayed unusual features, such as strong supercooling (EC and AA), specific interactions with PLGA (NMA), or different crystal structures (DMC), had a significant effect on the final
structures. Thus, a wide range of easily controllable structures can be conveniently obtained by the selection of a solvent, quenching strategy and removal process.

Micro-CT analysis, while providing important information about the macroporous architecture of scaffolds, has a number of limitations when characterizing scaffolds with significant micrometer-scale structure. The highest spatial resolution of the instrument used in this study gives a voxel size of 5 µm, which is significantly larger than many of the features observed via SEM. For example, in scaffolds produced using vacuum freeze-drying of DO and AA the internal wall structures are often <3 µm thick, while most of the micropores created during the leaching process have dimensions less than 5 µm. These features are impossible to resolve accurately using this method. In addition, the very low X-ray absorption of most polymeric materials leads to low contrast images and, hence, poor separation of air and solid during the reconstruction process. Thus, while the surface area/volume ratios shown in Table 2 are believed to be severe underestimates of the true values and the porosity values are somewhat unreliable, the values determined for the degree of anisotropy are believed to be reasonable estimates of the true values. However, this minimum value for the surface area/volume ratio is still very high, indicating the very large amount of available surface area within these scaffolds.

While this study was carried out using PLGA as a model polymer, it can be seen that in the absence of specific, strong solvent–polymer or polymer–polymer
interactions, the final structure obtained is almost exclusively dependent on the solvent type, quenching strategy and solvent removal strategy. Thus, a similar methodology may be used to form controlled structures from many other polymer types. For instance, synthetic polymers such as the lactide/glycolide homo- and co-polymers \[5, 10, 30\], polyurethanes \[31\], nylon 12 \[32\], polyethylene and polypropylene \[11\] and poly(\(\varepsilon\)-caprolactone) and its co-polymers \[33\], as well as natural polymers, such as collagen \[34\] and chitosan \[35\] may be treated via an analogous method. In many of these cases, structures have been reported which are very similar to the structures developed here.

The Young’s modulus in the axial direction was measured for the most promising scaffolds (Fig. 10). It is immediately apparent that the scaffolds with random structure had a higher modulus than the corresponding scaffolds with directional pore architecture. In the case of DO, the leaching process leads to an almost 10-fold drop in modulus compared to the vacuumed equivalent, while retaining excellent mechanical integrity. This would appear to be due to the softening and re-arrangement of the polymer structure during leaching, as observed by SEM (Fig. 6). By comparison, normal human fat has a Young’s modulus of approx. 0.02 MPa \[36\].

In the case of DMC, the Young’s modulus of the slow-quenched scaffolds was significantly lower than that of the quick-quenched samples. This correlates well with the structures observed by SEM: in this case the QQ led to a structure with more connections in the axial direction compared to the SQ, which was made up of very large parallel plates with widely-spaced axial struts. Notably, the structures formed using DMC were very springy compared to the other solvents, and were able to recover their original shape almost completely after multiple compression cycles at up to 50% axial strain (not shown). This is a potentially very useful property for soft tissue engineering applications.

The amount of solvent remaining in the final product is obviously a very important issue in gaining regulatory approval. Scaffolds prepared by the traditional method of freeze-drying using DO without further washing had a very large amount of DO residue, almost 3% of the scaffold mass, in agreement with previous reports in the literature \[13\]. Washing for 6 h with regular changes of water reduced this level to 600 ppm, an approx. 50-fold reduction. By comparison, the largest concentration of solvent measured in a leached scaffold was only 20 ppm, 30-fold lower still. Scaffolds prepared by vacuum freeze drying using DMC had a residual solvent concentration of only 21 ppm, even without further washing. These concentrations are all extremely low, well below the levels required by the standard for human pharmaceutical applications \[37\].

**CONCLUSIONS**

In this study, we have demonstrated the use of a number of ‘non-traditional’ solvents for the TIPS process. Solvents were selected primarily on the basis of predicted PLGA solubility and physical properties to allow convenient processing. The
solvents were further selected during the study based on toxicity and the mechanical integrity of the final scaffolds.

The effects of solvent type, cooling profile and removal method on the final scaffold structure were investigated using scanning electron microscopy (SEM) and micro-computed tomography (micro-CT). However, the use of micro-CT in analysis of scaffolds of the type produced here was found to be questionable. The removal of solvent via leaching into water led to a highly microporous structure within the walls of the macropores, which can be expected to dramatically change the surface roughness and diffusion properties within these scaffolds.

The modulus of the produced scaffolds ranged over an order of magnitude, depending on the production process, and correlated well with the morphology observed via SEM and micro-CT.

Of the solvents tested, ethylene carbonate, dimethyl sulphoxide and dimethylcarbonate stand out as the most promising non-toxic alternatives to dioxane for the production of tissue engineering scaffolds via the TIPS process. These solvents perform extremely well in terms of removability, convenient control of structure, and structural integrity of the final scaffolds.

This study has shown that in most cases, the solvent properties and quenching strategy, rather than the polymer/solvent or polymer/polymer interactions, have the most impact on the final structure. Thus, it appears that the methods and many of the solvents described here may be applied to a range of other polymer systems to create similar structures.

It has been shown that not only pore size, but also pore architecture have a significant effect on the attachment, migration, proliferation and differentiation of cells within a porous structure [3, 6, 7]. In addition, it has also been shown that surface roughness on the order of hundreds of nanometers to a few micrometers significantly affects cell behavior [38]. While the exact relationships are as yet unclear, the scaffolds produced here can be tuned to cover a very wide range of pore sizes, architectures and surface morphologies. Further studies will be required to decide the particular architecture used for any tissue engineering applications.

**Acknowledgements**

The authors acknowledge funding support of the Australian Research Council and the Particulate Fluids Processing Centre. We gratefully thank Mr. Roger Curtain from the University of Melbourne for kindly writing the computer programs for the temperature measurement and for his valuable assistance in the SEM characterization. We also thank Dr. Peter Self from the University of Adelaide and Mr. Paul Thomson from Thomson Scientific Instruments Pty. Ltd. for their technical support on micro-CT analysis.
REFERENCES

APPENDIX

Architecture — SEM

The observation of surface morphology of scaffolds made using DMO and AA under quick quench (QQ) conditions is shown in Fig. A1. Scaffolds made under QQ conditions have pore sizes ranging from about 50 µm to less than 10 µm.

The observation of this region of scaffolds made using DMO, DMSO, DO, AA is shown in Fig. A2. In general, scaffolds made under QQ conditions (a, b, d) showed directionally distributed, fibre-like structures, with alignment facing to the centre, while scaffolds made under slow quench (SQ) conditions (c, e), showed relatively isotropic structures with pore sizes ranging from approx. 10 µm to 50 µm. Scaffolds made using DMO or AA under SQ conditions lacked mechanical integrity and, hence, were not studied further.

Figure A3 shows the central region of the cross-section at half height of leached scaffolds. Again, the scaffold architecture was dependent on the solvent. While scaffolds made with DMSO, DO and DMC under QQ conditions (d, f, i) retained their directional architecture throughout the cross-section, scaffolds made with DMO and EC via the QQ method, and AA (a, b, h) showed less directional, more random structures in the interior. For all solvents except DMC, the central structure made under SQ conditions (c, e, g) was characterized by randomly-distributed pores of approx. 200 µm. Scaffolds made with DMC under SQ conditions retained their plate-like structure throughout the cross-section.

Figure A1. SEM observation of surface morphology of leached scaffolds. (a) DMO QQ, (b) AA QQ. Scale bar = 50 µm.
Figure A2. SEM observation of the outer region of the cross-section at half height of leached scaffolds. (a) DMO QQ, (b) DMSO QQ, (c) DMSO SQ, (d) DO QQ, (e) DO SQ, (f) AA QQ, (*) DMO and AA SQ, not imaged. Scale bar = 500 µm.

Figure A4 shows the SEM observation of the microporous wall structures formed during the leaching process. Scaffolds were made using DMO, EC, DO and AA. For all scaffolds leached in water, the walls of the macropores were themselves highly porous, with micropores ranging from <500 nm to a few micrometers forming a honeycomb structure. Correspondingly, the surfaces of the walls displayed high levels of micrometer-scale roughness.
Figure A2. (Continued).
Figure A3. SEM observation of the central region of the cross-section at half height of leached scaffolds. (a) DMO QQ, (b) EC QQ, (c) EC SQ, (d) DMSO QQ, (e) DMSO SQ, (f) DO QQ, (g) DO SQ, (h) AA QQ, (i) DMC QQ, (j) DMC SQ, (*) DMO and AA SQ, not imaged. Scale bar = 200 µm.
In general, the outer surfaces of vacuumed scaffolds (Fig. A5) displayed a more regular, compact wall structure than the corresponding leached scaffolds (Fig. 4). Scaffolds made with AA in particular displayed a much more regular structure in vacuumed scaffolds compared to their leached counterparts. Scaffolds made with DO via SQ and vacuum freeze-drying had a thin skin covering many of the surface pores, which was absent in the corresponding leached scaffolds. Vacuumed DMC scaffolds were almost identical to their hexane-leached counterparts.

Figure A6 shows the structure of the central region of the cross-section at half height of vacuumed scaffolds. Compared to the corresponding leached scaffolds, the structure was characterised by much more regular, well-defined features, thinner, non-porous walls and smaller pore sizes. Scaffolds made using DO under SQ conditions (b) showed a randomly distributed cell-like structure with pore sizes of approx. 50–100 µm, slightly smaller than in the leached samples. Scaffolds made
using DMC displayed a similar layered structure to the outer region; however, the average sheet size was smaller and the average spacing between sheets increased to 50–100 μm. Since the structure of scaffolds made using DMC via vacuum freeze-drying was essentially identical to those made via leaching into hexane, the latter were not considered for further analysis. Morphologically, there was very little difference between the centre and outer region of the scaffolds made using AA.

Architecture — micro-CT

Micro-CT 3D reconstruction of PLGA scaffolds was used to display an overview of the macroporous architecture of the scaffolds. The structures determined via micro-CT agreed well with SEM images of scaffolds produced via the same method. Figure A7 shows examples of reconstructed cubic sections from leached scaffolds made using EC, DMSO, DO and DMC (leached in hexane) under QQ (a) and SQ (b) conditions. Figure A8 shows the reconstructed vacuum-dried scaffolds made using DO. In general, the directional architecture was observed in the scaffold made under
Figure A4. (Continued).

QQ conditions. Relatively random structure was observed in the scaffold made using SQ conditions.
**Figure A5.** SEM observation of the surface of vacuumed scaffolds. (a) DO QQ, (b) DO SQ, (c) AA QQ, (d) DMC QQ, (e) DMC SQ, (*) AA SQ, not imaged. Scale bar = 20 µm (a), 200 µm (e), 50 µm (all other images).
Figure A6. SEM observation of the centre region of the cross-section at half height of vacuumed scaffolds. (a) DO QQ, (b) DO SQ, (c) DMC QQ, (d) DMC SQ, (e) AA QQ, (*) AA SQ, not imaged. Bar = 200 µm.
Figure A7. Micro-CT 3D reconstruction of leached 5% (w/v) PLGA scaffolds. (a) EC QQ, (b) EC SQ, (c) DMSO QQ, (d) DMSO SQ, (e) DO QQ, (f) DO SQ, (g) DMC QQ, (h) DMC SQ. The side length of the cubes is approx. 750 µm.
Figure A7. (Continued).
Figure A8. Micro-CT 3D reconstruction of vacuum-dried 5% (w/v) PLGA scaffolds. (a) DO QQ, (b) DO SQ. The side length of the cubes is approx. 750 µm.

Figure A9. SEM observation of 10% (w/v) PLGA scaffold structure formed using NMA as a solvent. A large number of microbeads with diameters ranging from a few to 10 µm were observed to be attached to the fiber surfaces in the scaffolds made from 10% PLGA.