PREPARATION OF THREE-DIMENSIONAL POLY(ε-CAPROLACTONE) POROUS TISSUE ENGINEERING SCAFFOLDS BY A COMBINATION OF MICROCELLULAR INJECTION FOAMING AND POLYMER LEACHING

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Abstract

Three-dimensional (3D) interconnected porous poly (ε-caprolactone) (PCL) scaffolds with desirable pore sizes and porosities have been prepared by utilizing both microcellular injection foaming and polymer leaching techniques. The incorporation of water-soluble poly(ethylene oxide) (PEO) served as a porogen to improve the porosity and interconnectivity. Highly oriented and elongated pore structures were obtained from the scaffolds via microcellular injection foaming. It was found that the pore diameter variety and the porosity increased with PEO. The compression modulus of the porous PCL scaffold decreased from 68.2 MPa for neat PCL to 46.7 MPa for 50%PCL (i.e., 50%PCL/50%PEO blend by volume) with an increase in porosity, which may render these scaffolds suitable for a variety of medical and tissue engineering applications. 3T3 fibroblast cell culture was performed to confirm the biocompatibility and cell viability of the scaffolds, which revealed that the cells proliferated the best on the 50% PCL scaffolds, as compared to the other three scaffolds, due to the more elongated and spindle-shaped pore structures, indicating favorable cell–scaffold interactions. Therefore, this novel method offers an effective means for scalable fabrication of tissue engineering scaffolds.

Introduction

Tissue engineering is aimed at developing biological substitutes that restore, maintain, or improve tissue function [1]. So far, tissue engineering has already been used to improve the recovery of different types of tissues such as skin, bones, blood vessels, and nerve conduits [2]. Currently, the main challenge for tissue engineered scaffolds is the need to mass produce three-dimensional (3D), fully, interconnected, highly porous scaffolds with suitable surface properties that promote the biological activities of both seeded and native cells. However, the porosity and mechanical properties, which are both important traits of tissue engineering scaffolds, are closely interrelated [3].

Several different techniques have been established to create open pore structures using biodegradable polymers. The most common 3D biodegradable polymer scaffolding fabrication techniques include solvent extraction [4], particulate leaching [5], microsphere sintering [6], gel casting [7], phase separation [8], 3D printing [9], and gas foaming [2].

Polymeric foams have been attracting more and more attention recently due to their advantages, such as reduced material consumption; reduced part weight; and improved heat, impact, and acoustic absorption. Microcellular injection foaming (MIF) [10] is attracting increasing attention from researchers because it does not require an organic solvent and is environmentally friendly. Additionally, it is a foaming method that is able to mass produce biobased polymeric foams. MIF has high productivity and it is easy to injection mold the scaffolds with the required geometries and shapes. Moreover, it is a relatively new fabrication method for tissue engineering, and the know-how needed to control and balance the scaffold properties and microstructure has yet to be fully developed. However, this process is limited to a certain pore size, pore variety (size and shape), and porosity and, most of the time, it generates a closed cellular structure within the molded component, precluding it from tissue engineering scaffold applications that require an interconnected pore structure. Fortunately, this shortcoming can be circumvented using a combination of methods.

Polymer leaching (PL) is a cost-effective technique for producing porous scaffolds that can yield fully inter-connected porous networks where interconnections are characterized by channels, instead of small pores, that connect larger pores. This method mainly focuses on blending a water-soluble polymer with the scaffold matrix material; a porous structure can then be created after leaching the water-soluble phase, such as poly(vinyl alcohol) (PVA) or poly(ethylene oxide) (PEO) [11, 12]. Combining PL and MIF can yield better pore size control, thus enabling the fabrication of scaffolds with multimodal pore size distributions.

In this study, PCL is blended with PEO, which is also biodegradable, biocompatible, and readily dissolved in...
water [13]. PEO does not require potentially toxic organic solvents for dissolution. Additionally, PEO itself is non-toxic to cells; therefore, residual polymer should not affect cell viability. Following the melt blending of PCL with PEO using a twin-screw extruder, the MIF technique is applied to create a certain extent of open pores and highly oriented and elongated pore structures. Subsequently, water-soluble PEO is dissolved to enhance the overall pore size and porosity. However, to date, there have been no studies that research the combination of MIF and PL to fabricate PCL porous scaffolds. The purpose of this research is to evaluate the feasibility of combining these techniques for the preparation of highly interconnected three-dimensional porous scaffolds. Scaffold morphology, mechanical properties, and biocompatibility were investigated as well.

**Materials**

PCL (Capa 6500 from Perstorp U.K. Ltd.), a biocompatible and biodegradable polymer, was used as the matrix material in this study. It has a melt flow index of around 7 g/10 min (160 °C/2.16 kg). It has a glass transition temperature of −60 °C, a melting temperature of 58–60 °C, and a specific gravity of 1.10. PEO, with an average molecular weight of 100,000, was purchased in powder form from Sigma–Aldrich. It has a specific gravity of 1.13 and a melting temperature of 65 °C. Nitrogen (N₂) was chosen as the blowing agent for the MIF process. It has a lower solubility in most polymers and yields a finer porous structure in the foaming process [14].

**Scaffold Fabrication**

The PCL and PEO were dried in a vacuum oven at 45 °C for 24 h prior to compounding. Materials with a variety of volume formulas—PCL (100%PCL), 90%PCL (90%PCL/10%PEO), 70%PCL (70%PCL/30%PEO), and 50%PCL (50%PCL/50%PEO)—were compounded with a twin-screw extruder (Leistritz ZSE 18 HPe) at 120 °C (the die temperature) at a screw speed of 120 rad/min, followed by circular water cooling and granulation. As a reference, neat PCL pellets were also run through the extruder using the same conditions with the same thermal history as the PCL/PEO blends.

MIF can produce foamed parts with a specific geometry. The pre-blended pellets were dried in a vacuum oven at 45 °C for 24 h to remove any moisture before being used for MIF. An injection molding machine (Arburg Allrounder 320S) with a standard ASTM D638 Type I tensile bar cavity was used to produce solid tensile bar samples. The injection molding machine, which had a 25 mm diameter screw and a mold temperature controlling device, was equipped with a supercritical nitrogen (scN₂) supply system (MuCell Trexel, Inc.) that enabled precise control of the gas dosage by adjusting the gas flow rate and valve open time. The MIF processing parameters are listed in Table 1.

Next, the prepared scaffolds were cut from the middle section of the MIF tensile test bars. In this case, the resulting foamed PCL/PEO samples were immersed in deionized water to leach out the PEO. The water was changed every 12 h up to a constant weight was reached and then a wet sample was obtained. The whole process of preparing foamed PCL scaffolds is shown in Fig. 1.

Table 1. Experimental conditions for the MIF process.

<table>
<thead>
<tr>
<th>Molding parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nozzle temperature (°C)</td>
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<tr>
<td>Mold temperature (°C)</td>
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<tr>
<td>Injection speed (cm²/s)</td>
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<td>Injection pressure (bar)</td>
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<tr>
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<td>Loading of SCF (%)</td>
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</tr>
<tr>
<td>SCF dosage time (sec)</td>
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</tbody>
</table>

**Figure 1. Schematic illustration of this novel PCL tissue scaffold fabrication method.**

**NIH 3T3 Fibroblast Cell Culture**

Microcellular injection foamed samples that were leached of PEO were chosen for cell culture to investigate the biocompatibility of, and cell viability on, the four prepared PCL foams for potential tissue engineering scaffold applications. Briefly, 3T3 cells were maintained prior to testing on 6-well tissue culture-treated polystyrene plates (BD Falcon). The microcellular injection foamed samples that were leached of PEO were cut into cross-sectional pieces of about 10 mm × 8 mm × 2 mm at the center of the tensile test bar and were placed in 24-well tissue culture-treated polystyrene plates after sterilization with UV light for 1 h (30 min each side). 3T3 cells were treated with ethylenediaminetetraacetic acid (EDTA) for 5 min and washed with phosphate-buffered saline (PBS) prior to seeding. Cells were then seeded at a density of 1.25×10^5 cells/cm² in the high-glucose 3T3 medium. Spent medium was aspirated and replaced with 1 mL of fresh medium daily for screening samples.
Characterization

Mechanical Properties

The mechanical properties of the scaffolds were characterized via compression tests of the foamed PCL scaffold samples after leaching PEO and being thoroughly dried. These tests were performed using a universal testing machine (Instron 5967, USA) with a 250 N load cell. The test rectangular samples cut from the middle of the foamed tensile bars were tested following the standard test method (ASTM D695). All samples were compressed to 50% strain at a speed of 5 mm/mm. Statistical results were the average of the five samples. All of these tests were carried out at ambient temperature (23 °C). The compressive modulus was evaluated from the entire linear region of the compressive stress–strain curve.

Scanning Electron Microscopy (SEM)

The microstructure of microcellular injection foamed samples before and after leaching PEO were evaluated using a scanning electron microscope (NeoScope JCM-5000) with an accelerating voltage of 10 kV. All specimens were frozen in liquid nitrogen and fractured by two clamps to expose the cross section at the middle of the molded tensile bars. SEM observations were made after sputtering the samples with a thin film of gold for 45 s.

Porosity Measurements

Microcellular injection foamed samples post leaching were trimmed into rectangles and their porosities were determined using Eq. (1) by weighing the samples and measuring their dimensions to obtain their volume. The porosity was the mean value of five samples,

\[
\text{Porosity} = \frac{V_{\text{m}} - W_{\text{m}}}{V_{\text{m}}} \times 100\%
\]

where \(W_{\text{m}}\) was the measured weight of the scaffold, \(\rho\) was the density of PCL, and \(V_{\text{m}}\) was the volume of the scaffold sample.

3T3 Fibroblast Cell Viability

Fibroblast cell viability was determined 3 days and 10 days after seeding. Viability was assessed via a Live/Dead Viability/Cytotoxicity Kit (Invitrogen). The stain utilized green fluorescent Calcein-AM to target esterase activity within the cytoplasm of living cells, and red fluorescent ethidium homodimer-1 (EthD-1) to indicate cell death by penetrating damaged cell membranes. Stained cells were imaged with a Nikon Eclipse Ti Microscope with an attached Photometrics CoolSNAP HQ2 camera. Nis-D Elements Advanced Research v.3.22 software was used for image analysis.

Results and Discussion

Morphology of the Scaffolds before Leaching PEO

Figure 2 shows the morphology of injection foamed PCL and PCL/PEO samples before leaching PEO. It was found that PCL exhibited an elliptical pore structure, while the pores of the PCL/PEO samples were elongated in the thickness direction of the tensile bar sample with a high length-to-width ratio, especially for the 50%PCL samples. To further specify the elongation of the pores, the pore diameters along the long axis were measured, and the size distributions are shown to the right of the corresponding SEM micrographs. The statistical results showed that PCL had a relatively concentrated pore size distribution in the sample’s thickness direction, with pore sizes ranging from 54.4 to 232.3 µm. This distribution became wider and shifted to higher values as the PEO content increased in the PCL, indicating that the addition of PEO assisted in the formation of elongated pores. The effect of PEO on PCL MIF is significant, and this highly elongated anisotropic pore structure may provide some unique properties for the PCL/PEO foamed samples.
The mean long axis pore size was measured and is shown in Figure 3. It can be seen that the mean long axis pore size increasing from 132.2 µm to 460.1 µm, thus indicating that the shape of the pore became longer. This might have been due to the viscosity of the PCL/PEO samples decreasing with higher PEO content, thus resulting in pores that grew more readily in the thickness direction of the tensile bars. As the injection foaming conditions were the same for all of the samples, the pore expansion mainly depended on the melt strength of the samples. Since the melt strength of the matrix was weakened by the N₂-induced plasticization effect, the matrix became too weak to sustain pore expansion, and the pores had a tendency to break at the pore strut wall, which resulted in larger, open pores in the PCL/PEO scaffolds.

![Figure 3. Effect of PEO content on the mean long axis pore size of foamed PCL samples before leaching PEO.](image)

**Morphology of the Scaffolds after Leaching PEO**

For tissue engineering scaffold applications, the pore structure and porosity are important parameters, and they must match the type of tissue being regenerated [15]. Thus, another key step to enhancing the controllability of the pore structure is polymer leaching. Figure 4 shows the morphology of injection foamed PCL and PCL/PEO samples after leaching PEO. The images on the right are the magnification of the corresponding images on the left. PCL (Fig. 4 (a)) presented an almost closed-pore structure. However, 90%PCL, 70%PCL, and 50%PCL scaffolds exhibited an interconnected pore structure through openings (holes) in the pore walls. Furthermore, the connectivity of the pores was enhanced at higher PEO ratios. Another feature that is noteworthy for the pore structure is that the opening between the pores was relatively large, interconnecting pores both large and small, especially for the 50%PCL sample (Fig. 4 (d)). Interestingly, the 70%PCL and 50%PCL samples had pores with an obvious bimodal cellular morphology. That is, the tiny pores and large pores connected to form the bimodal structure. This will facilitate the migration of seeded cells from one pore to another, as well as the cell penetration from the scaffold surface to the internal portion of the scaffold.

![Figure 4. SEM images of foamed PCL scaffolds after leaching PEO: (a) PCL, (b) 90%PCL, (c) 70%PCL, and (d) 50%PCL. Subscript 2 images (scale bar: 200 µm) are enlarged images of subscript 1 images (scale bar: 50 µm).](image)

The statistical results of porosity are shown in Fig. 5. It was found that the porosity increased as the PEO content increased. Additionally, a maximum porosity of 89.5% was obtained for 50%PCL, thus indicating that the leaching of PEO combined with injection foaming enabled PCL to meet the requirements of tissue engineering scaffolds; that is, a highly porous and interconnected structure.

![Figure 5. The statistical results for porosity of microcellular injection foam ed PCL scaffolds after leaching PEO.](image)
Mechanical Properties

The mechanical properties of scaffolds are of crucial importance in tissue engineering applications. Compression tests were used to evaluate the mechanical strength of the tissue engineering scaffolds and the injection foamed PCL scaffolds after leaching PEO; see Fig. 6. PCL had a high compressive modulus and compressive yield stress. Conceivably, the compressive modulus and yield stress decreased as the leached PEO content increased. The compressive modulus ranged from 46.7 to 68.2 MPa, which meets the compressive modulus requirement of cancellous bone [2]. Thus, the PCL scaffolds mass produced by MIF with leaching afterward have great potential in meeting the mechanical property requirements for human tissues.

Cell Viability and Proliferation

The biocompatibility of the PCL scaffolds was tested by culturing 3T3 fibroblasts for 3 days and 10 days to explore the potential of these samples being used as tissue engineering scaffolds in biomedical applications. The 3T3 fibroblast cell morphology Day 3 and Day 10 results are shown in Figure 7. Figures 7 (a) through (d) show the Day 3 results. It is clear that fibroblasts attached and spread all over the scaffolds. In the fluorescence images, the green color indicates live cells and the red color represents dead cells. The fluorescence images show large quantities of live cells. PCL scaffolds (Fig. 7 (a)) show a large amount of live cells as compared to bare dead cells. It was also found that the live cells increased and the dead cells decreased for 90%PCL and 70%PCL scaffolds as compared with neat PCL samples, thus indicating good biocompatibility between the substrate and the cells. It is worth noting that the 50%PCL sample had almost all live cells and no dead cells, demonstrating that 50%PCL had the best biocompatibility. Moreover, the dispersion of live cells was the most uniform for the 50%PCL sample. This could be due to the maximum porosity and pore diameter variety of the 50%PCL sample (recall Figs. 4 and 5).

The Day 10 fluorescence images (Figures 7 (e) through (h)) show that very few dead cells were present on all four scaffolds. There were more and denser cells on all scaffolds compared to Day 3. Most cells were still alive, indicating that all scaffolds showed good biocompatibility. The trend of the biocompatibility of Day 10 was the same as Day 3. In addition, the media was relatively clear, suggesting that not many dead cells were washed away during periodic culture media replacement. It was observed that live cells continued to proliferate on the scaffolds which indicated that the scaffolds provided a suitable environment for cells to grow.

Therefore, it was concluded in this study that porosity and pore diameter variety can enhance the biocompatibility of PCL scaffolds. The results confirm the positive advantage of combining MIF and PL on cell survival and cell growth.

Conclusions

PCL porous scaffolds with porosity levels up to 89.5% and a dual pore size distribution were obtained by a combination of microcellular injection foaming (MIF) and polymer leaching (PL) techniques. Compared to conventional salt particle leaching, this new fabrication technique allows for better control of pore interconnectivity by the creation of a fully interconnected porous network. Supercritical N₂ was used to generate the micropores inside of the PCL matrix. PEO leaching was used to further enhance the interconnectivity of the scaffolds. The PEO leaching technique also allowed for the porosity range to be widened from 58.3% for PCL samples to 89.5% for 50%PCL, while still maintaining good mechanical integrity. Furthermore, a greater variety of pore shapes and sizes became more obvious for 50%PCL scaffolds with this novel method. As expected, the compression moduli of the porous PCL materials decreased with increased porosity. The values varied form about 68.2 to 46.7 MPa, which may render these scaffolds suitable for multiple tissue types in a variety of medical and tissue engineering applications, including load bearing applications. 3T3 fibroblast cell culture experiments demonstrated that the method used in this
study significantly enhanced the biocompatibility of the PCL scaffolds, and that the 50%PCL scaffold had the highest cell adhesion, cell viability, and cell proliferation of the PCL scaffolds tested. The novel solvent-free approach proposed in this study provides a new, green tissue engineering scaffold fabrication method that has the potential to mass produce tissue scaffolds and be used for multiple tissue types in a variety of medical and tissue engineering applications.

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References